Indian Journal of Clinical Biochemistry, 2003, 18 (2) 136-149

STUDIES ON BIOCHEMICAL CHANGES WITH SPECIAL REFERENCE TO OXIDANT AND ANTIOXIDANTS IN MALARIA PATIENTS

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ABSTRACT

Oxidative stress plays an important role in the development of malarial anemia. The present study was undertaken to study the role of oxidant and antioxidants in the patients of *Plasmodium falciparum* malaria (n = 25), *Plasmodium vivax* malaria (n = 25) as against the normal control subjects (n =25). The parameters included are the hematological [hemoglobin, erythrocyte adenosine deaminase (ADA) activity, ADP – induced platelet aggregation] and serum total lipid peroxide as an index of oxidative stress and antioxidants [erythrocytic superoxide dismutase (SOD) activity,serum vitamin E] & serum iron.

Significant alterations in all above parameters were noted in both groups of malaria patients as compared to control subjects. Maximum significant alterations in hematological parameters were noticed in *P. falciparum* infection as compared to *P. vivax* malaria ($p<0.001$). Substantial rise in serum total lipid peroxides and a significant reduction in antioxidants such as serum vitamin E and serum iron were noted in *P. falciparum* malaria as compared to *P. vivax* malaria ($p< 0.001$), whereas maximum decline in erythrocytic SOD activity was observed in *P. vivax* infection as compared to *P. falciparum* malaria ($p<0.05$). Follow-up examination revealed the restoration of the levels of all biochemical parameters to the normal level after 20 days of antimalarial therapy.

The study specified severity of *P. falciparum* malaria and also functional duality of oxidant.

KEY WORDS

Hematological Parameters, Lipid Peroxidation, Antioxidants, Malaria.

INTRODUCTION

Formidability of malarial infection principally lies in the development of anemia. Along with this other hematological changes such as thrombocytopenia, increased erythrocytic adenosine deaminase activity are also observed in malaria infected persons (1,2).

One of the major reasons for development of malarial anemia seems to be oxidative stress (3,4). Any infection, including malaria, activates the immune system of body thereby causing release of reactive oxygen species as an antimicrobial action. In addition to host's immune system, malarial parasite also stimulates certain cells in production of reactive oxygen species thereby resulting in hemoglobin degradation (3,5).

Macrophage generated reactive oxygen species are known as nonspecific effector molecules in their defence armoury, may not only damage the parasitized red blood cells but also 'innocent bystanders' such as non- parasitized red blood cells. The accumulation of organic peroxides and oxidation of membrane lipids place a stress on cellular vitality ultimately leading to destructive effects on the cell. Earlier research workers reported about increased lipid peroxidation in malaria patients, particularly in *P. falciparum* infection (2,3,6). Instantaneous reduction in antioxidant potency in tandem with increased lipid peroxidation is also observed to be equally accountable for development of oxidative stress in malaria patients (3,7,8,9).

Thus, the specific goal of the present work was to study the role of oxidative stress and antioxidative status in malaria.

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MATERIALS AND METHODS

The study included fifty malaria patients diagnosed on the basis of clinical findings and positive peripheral blood smear for malarial parasite. The twenty-five healthy control subjects were matched for sex & socioeconomic status. All hee subjects undertaken for study were between the age group of 20-45 yr. This age group was selected to avoid any age dependent changes in relation to oxidative stress and antioxidative capacity. The malaria patients were grouped on the basis of presence of particular species of malarial parasite as patients with *P.falciparum* malaria (n = 25) and patients with *P. vivax* malaria (n = 25). Blood samples from these patients were collected before giving any antimalarial therapy at the time of hospitalization and the hematological parameters, oxidant and antioxidants were estimated.

I) Hematological parameters

Blood hemoglobin was measured by Sahli's method (10). ADP – induced platelet aggregability was determined by the method of O' Brien (11). Erythrocytic adenosine deaminase activity was estimated by modified method of Giuseppe Giusti described by Severini (12).

II) Oxidant

Serum total lipid peroxides were quantified by Kei Satoh's method (13).

III) Antioxidants

Erythrocytic superoxide dismutase activity was measured by the method of Marklund and Marklund (14). Serum vitamin E was assessed by the method of Baker and Frank (15). Ramsay's dipyridyl method was applied for determination of serum iron (16).

During follow-up study above biochemical parameters were again investigated after three and twenty days of antimalarial therapy to assess their prognostic significance.

Statistical analysis

Statistical analysis was carried out by using student's paired and unpaired 't' test. The $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The findings of the present work in malaria patients are summarized in three parts.

I) Alterations in hematological parameters

A significant decline in hemoglobin concentration was noticed in all untreated malaria patients as compared to control subjects ($p < 0.05$) (Table 1, 2). Maximum reduction in hemoglobin concentration was observed in *P. falciparum* infection as compared to *P. vivax* infection ($p < 0.001$) (Table 3).

Hemoglobin is the major food material of malarial parasite. By degradation of hemoglobin, the malarial parasite fulfills its need of amino acids (17). Loria *et al* (5) observed that the mature stage parasitized erythrocyte contain only 25% of the total hemoglobin level present in a normal nonparasitized red blood cell. Both the factors i.e. hemolysis and hemoglobin degradation by malaria parasite contribute for anemic status in malaria patients.

Present analysis also specified dramatic increase in erythrocyte adenosine deaminase activity in untreated malaria patients as compared to normal subjects ($p<0.05$) (Table 1, 2). Maximum rise in erythrocyte adenosine deaminase activity was noted in *P. falciparum* malaria as compared to *P. vivax* infection ($p<0.001$) (Table 3). Daddona *et al* (18) found that adenosine deaminase deficient erythrocytes, after invasion by *P. falciparum*, showed about two fold increase in adenosine deaminase activity over the normal range. Their further investigations pointed out that the increased expression of erythrocytic adenosine deaminase activity was the result of production of parasite specific adenosine deaminase. Malarial parasites are unable to carry out *de novo* purine synthesis which is essential for nucleic acid synthesis during their proliferation. For this purpose hypoxanthine, required for purine salvage pathway, is obtained by two ways. First is the parasitized erythrocytes' external milieu and second one is the adenosine metabolism. Adenosine metabolism comprises the enzymes adenosine deaminase and purine nucleoside phosphorylase. The presence of high activity of parasite specific ADA in *P. falciparum* insinuates a unique metabolic role of this enzyme in growth and proliferation of intraerythrocytic malaria parasite. Metabolism of adenosine to obtain hypoxanthine seems to have a selective advantage over the another way as the host erythrocytes does not compete for utilization of hypoxanthine (18). The concurrent increase in erythrocytic adenosine deaminase activity in tandem with reduced

hemoglobin concentration represented by a negative correlation between these two parameters exhibits rapid proliferation of malaria parasite ($r = -0.720$) (Figure1).

In addition to these changes, the present analysis also showed the increased ADP-induced platelet aggregation in untreated *P. falciparum* malaria patients as compared to controls and *P.vivax* malaria patients ($p < 0.001$) (Table 1-3). Maximum induction of ADP- induced platelet aggregation was found in *P. falciparum* infection as compared to *P. vivax* malaria which reflects the vulnerability of these patients to develop platelet aggregation induced complications and endothelial injury evidenced by establishment of significant negative correlation between ADP induced platelet aggregation and hemoglobin concentration ($r = -0.637$) (Figure 2). Inyang *et al* (19) allowed the interaction of normal platelets with *P. falciparum* and found significant increase in aggregation response of the platelets to external stimuli such as ADP, which exhibits the platelet dysfunction. Another reason behind the increased platelet aggregation in malaria patients might be upsurged lipid peroxidation.

II) Enhancement of oxidant status in malaria patients

Momentous upsurge in serum total lipid peroxides was noted in untreated malaria patients as compared to controls ($p < 0.01$) (Table 1,2). The patients with *P. falciparum* infection epitomized highest levels of lipid peroxides as compared to *P. vivax* malaria patients ($p < 0.001$) (Table 3). The increased lipid peroxides was an ultimate toxic effect of upsurged reactive oxygen species production by immune system as well as synchronized release of O_2^- during hemoglobin degradation by malarial parasite. It has been shown that intact. *P. falciparum* trophozoite infected human red cells produce H_2O_2 and OH^- radical about twice as much as normal erythrocytes (2). *P. falciparum* infection can bring about membrane lipid peroxidation even in absence of oxidative stress generated by activated blood monocytes (7). Rath *et al* (6) reported an increased levels of serum lipid peroxidation in *P.falciparum* infected patients as compared to *P. vivax* infection . In addition to this, phagocytosis of malarial pigment or pigment containing erythrocytes by phagocytes,results in release of large number of reactive oxygen intermediates for a short period (4). Besides this, the plasma of malaria patients has been suspected to contain prooxidants. Hemoglobin can act as prooxidant by catalyzing the decomposition of lipid hydroperoxides enhancing the chain reaction (20).

On the other hand, the combination of hemoglobin and hydrogen peroxide are capable of accelerating lipid peroxidation . Excess hydrogen peroxide could also results in break down of heme and release of free iron ions, which in turn form OH via Fenton reaction (20). Increased adenosine deaminase activity brings about increased xanthine oxidase activity thereby representing another source of superoxide anion formation (2). Thus in unison, all these factors cause a substantial rise in lipid peroxidation, leading to oxidative stress in malaria. A negative correlation obtained between hemoglobin concentration and serum lipid peroxides in malaria patients ($r = -0.840$) (Figure 3) indicates a vital role of oxidants in malaria.

III) Depression of antioxidative capacity

Present analysis specified concurrent reduction in antioxidants in concert with the momentous increase in oxidative stress in untreated malaria patients. Due to synergistic functioning, the erythrocytic superoxide dismutase, serum α-tocopherol and serum iron were studied specifically, to evaluate antioxidant potential in malaria.

There was overall decline in erythrocytic SOD activity in malaria patients before treatment as compared to controls ($p < 0.05$) (Table 1, 2). As shown in Table 3, maximum decline in erythrocytic SOD activity was observed in *P. vivax* infection as compared to *P. falciparum* infection ($p<0.05$). The fact that oxidative drugs mediate parasitic clearance insinuates that the malarial parasite is also sensitive to oxidative stress (8). To counteract the oxidative damage the malarial parasites utilize the host's antioxidant defence such as erythrocytic SOD. Ranz & Meshnick (21) have shown that in addition to utilization of host SOD, P. *falciparum* also synthesizes the supplementary parasite specific SOD, which is cyanide insensitive, unlike to that of human SOD . The mature stage intraerythrocytic *P. falciparum* trophozoites contain four fold higher parasite specific SOD than the early developmental stages (9), whereas presence of endogenous SOD has not been observed in case of *P. vivax* (22). The antioxidant enzymes degraded by malarial parasite to derive amino acids, can not be replenished by red blood cells due to lack of protein synthesis which might be one of the reasons behind overall decrease

in erythrocyte SOD activity in malaria patients as compared to controls (3). A negative correlation was obtained between erythrocyte superoxide dismutase activity and hemoglobin concentration in malaria patients which indicated that at much higher parasitemia with lower hemoglobin level, the infected red cells may possess higher SOD activities than those in normal red cells (r= -0.504) (Figure 4).

Present study also specified significant reduction in serum vitamin E in untreated malaria patients as compared to controls ($p<0.001$) (Table 1,2). A significant decline in serum vitamin E concentration was noted in *P. falciparum* malaria as compared to *P. vivax* malaria ($p<0.001$) (Table 3).

The dwindle observed in vitamin E might be due to; i) its transfer to red blood cell membrane to counteract the increased oxidative stress during acute phase of disease by inhibiting membrane lipid peroxidation, ii) its increased utilization as plasma antioxidant and iii) the impaired release of vitamin E may occur during acute phase of disease (23).

The fall in vitamin E level failed to control platelet aggregation, as vitamin E is known to restrain platelet aggregation (24). Besides this the reduced vitamin E content of serum also augmented lipid peroxidation. In present analysis, the serum vitamin E level positively correlated with hemoglobin concentration & thus signified a vital role in malaria ($r=0.739$) (Figure 5).

In present work, serum iron was significantly reduced in all malaria patients as compared to normal subjects ($p< 0.001$) (Table 1,2). A considerable decline was observed in serum iron in *P. falciparum* malaria as compared to *P. vivax* malaria ($p<0.05$) (Table 3).

The major motive of diminution in serum iron might be iron sequestration in malarial pigment. Besides this hypoferraemia is a constant feature of infectious diseases. Decreased iron absorption from gastrointestinal tract during acute phase of malaria also contributes for decrease in serum iron (25). In malaria, serum iron seems to have a divergent role.

i) The decline in iron content may be responsible for lowered catalase activity, thus reduces the degradation of hydrogen peroxide.

ii) Iron ions are also essential for parasitic growth as they act as cofactor for various enzymes involving in parasitic metabolism and thus the reduced iron retards parasitic growth. Decreased iron also minimizes the iron mediated lipid peroxidation.

As seen in Figure 4, There is a significant positive correlation between serum iron and blood hemoglobin in untreated malaria patients ($r = 0.571$) (Figure 6) which indicates the malaria patients are prone to develop iron deficiency anemia. The malaria patients of both groups received a three day standard chloroquine treatment and in case of *P. vivax* malaria, primaquine was administered after chloroquine treatment to avoid relapses. Chloroquine interferes with carbohydrate metabolism by inhibiting both, the uptake of glucose and glycolysis and also causes accumulation of H_2O_2 in the acidic food vacuole of parasite (17,5). Besides this chloroquine also inhibits heme degradation thereby causing heme mediated damage to parasite (5).

During follow up study, after three days of antimalarial treatment it was noticed that there was increase in hemoglobin concentration and decrease in ADP induced platelet aggregation and erythrocytic adenosine deaminase activity due to chloroquine mediated parasitic clearance in all malaria patients as compared to the status before treatment.

After three days of chloroquine treatment a decline in serum total lipid peroxides was observed as compared to pre-treatment in malaria patients which might be due to reduction in reactive oxygen species generation by malarial parasite due to parasitic clearance. However, the substantial sustained increase in lipid peroxides in malaria patients as compared to control subjects might be the result of reactive oxygen species production by activated immune system. Besides this, the destructive action of chloroquine towards the malarial parasite is partly dependent on reactive oxygen intermediates (5). The present study did not show any considerable difference in the erythrocytic SOD activities in malaria patients before treatment and after three days of treatment. The rise in vitamin E level in malaria patients, after three days of treatment might be due to restorement of hepatic function (23), whereas hemolysis could be the possible cause of increased iron content after three days of treatment as compared to pre-treatment status.

After twenty days of antimalarial treatment, the levels of all biochemical parameters were restored to normal levels.

Finally, from these biochemical studies it can be concluded that the oxidative stress has a functional duality due to its involvement in tissue damage as well as in parasitic destruction. This study also reflects the severity of *P. falciparum* malaria.

However, the administration of vitamin E after adequate treatment for parasitic clearance may be fruitful to avoid malarial anemia, particularly in *P. falciparum* malaria. This can be confirmed by replicating the study with a larger trial.

Table 1
Depicting the status of hematological parameters, lipid peroxide and antioxidants in control subjects and *P.vivax* malaria patients.

Sr. No.	Paramenters	Control Group (normal subjects) n = 25	*P.vivax* malaria patients (n=25) Before treatment	After three days of treatment	After twenty days of treatment
I	Hematological Parameters				
a)	Hb [gm%]	13.5 ± 0.89	10.1 ± 0.97 **	11.27 ± 0.83 * +	12.3 ± 0.91 ● ++
b)	Platelet aggregation [%]	34 ± 3.5	46.69 ± 2.7 ●	40.64 ± 1.2 ● ◆	37.2 ± 4.1 ● ◆
c)	Erythrocytic ADA [U/gm Hb]	11.43 ± 0.71	14.38 ± 0.83 ***	12.97 ± 0.38 ** +	11.6 ± 0.31 ● +++
II)	Oxidant				
a)	Serum total lipid peroxides [nmol/ml]	1.9 + 0.12	3.67 ± 0.16 **	2.94 ± 0.14 * +	2.07 ± 0.11 ● +++
III)	Antioxidants				
a)	Erythrocytic SOD [U/gm Hb]	1271 ± 60	1073 ± 58.2 **	1130 ± 77.3 ● ◆	1222.7 ± 69 ● +
b)	Serum Vitamin E [mg%]	1.22 ± 0.046	0.930 ± 0.092 **	1.13 ± 0.026 * +	1.17 ± 0.083 ● +
c)	Serum Iron (µg%)	130.1 + 4.25	96.3 ± 3.45 **	112 ± 2.86 * +	122.4 ± 4.1 ● ++

Values are expressed as Mean ± S.D. n = number of subjects studied.

Comparison of all other groups with control group
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ● $p > 0.05$ or NS

Comparison of the patients before and after antimalarial treatment
+++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$, ◆ $p > 0.05$ or NS.

Table 2

Showing status of hematological parameters, lipid peroxide and antioxidants in control subjects and *P.falciparum* malaria patients.

Sr. No.	Paramenters	Control Group (normal subjects) n = 25	*P.falciparum* malaria patients (n=25) Before treatment	After three days of treatment	After twenty days of treatment
I	Hematological Parameters				
a)	Hb [gm%]	13.5 ± 0.89	7.27 ± 0.23 ***	8.83 ± 0.31 *** ++	11.32 ± 0.41 ● +++
b)	Platelet aggregation [%]	34 ± 3.5	76.19 ± 2.4 ***	66.12 ± 1.67 ** ++	42 ± 4.3 ● +++
c)	Erythrocytic ADA [U/gm Hb]	11.43 ± 0.71	16.63 ± 0.78 ***	14.21 ± 0.31 *** ++	12.5 ± 0.61 ● +++
II)	Oxidant				
a)	Serum total lipid peroxides [nmol/ml]	1.9 ± 0.12	5.42 ± 0.18 ***	4.60 ± 0.12 *** +	2.15 ± 0.14 ● +++
III)	Antioxidants				
a)	Erythrocytic SOD [U/gm Hb]	1271 ± 60	1133 ± 51 *	1230 ± 56 ● ◆	1250 ± 72 ● ++
b)	Serum Vitamin E [mg%]	1.22 ± 0.046	0.706 ± 0.065 ***	0.862 ± 0.079 *** ++	1.13 ± 0.103 ● +++
c)	Serum Iron (μg%)	130.1 + 4.25	81.93 ± 3.17 ***	97.91 ± 4.41 ** +	115.1 ± 8.6 ● +++

Values are expressed as Mean ± S.D. n = number of subjects studied.

Comparison of all other groups with control group
*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ● $p > 0.05$ or NS

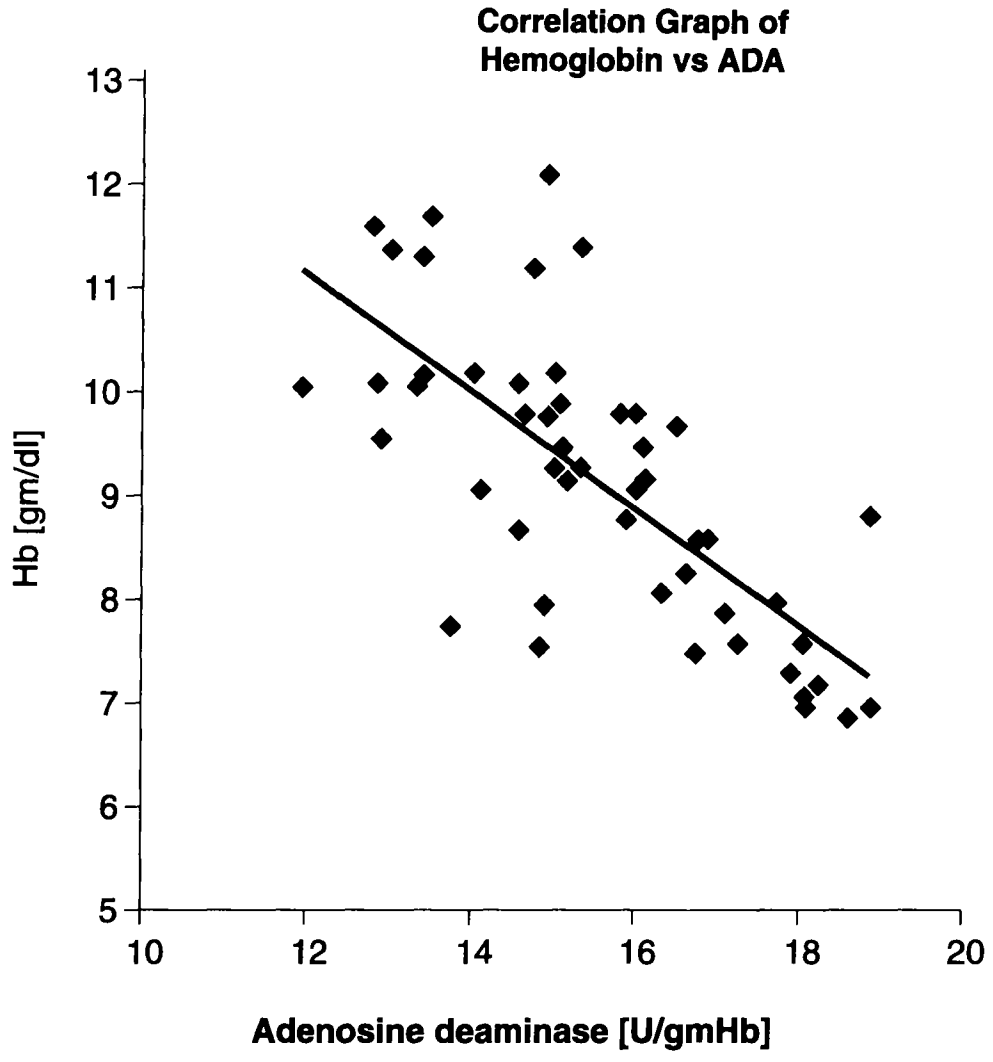
Comparison of after treatment groups with before treatment group
+++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$, ◆ $p > 0.05$ or NS.

Table 3
Indicating comparison status of hematological parameters, lipid peroxidesand antioxidants inbetween *P.vivax* and *P. falciparum* malaria patients.

Sr. No.	Paramenters	*P.vivax* Malaria Patients (n=25) Mean+S.D.	*P.falciparum* Malaria Patients (n=25) Mean+S.D.	Comparison *P.vivax* & *P. falciparum* malaria patients (p value)
I	Hematological Parameters			
a)	Hb [gm%]	10.1 ± 0.97	7.27 ± 0.23	< 0.001
b)	Platelet aggregation [%]	46.69 ± 2.7	76.19 ± 2.4	< 0.001
c)	Erythrocytic ADA [U/gm Hb]	14.38 ± 0.83	16.63 ± 0.78	< 0.001
II)	Oxidant			
a)	Serum total lipid peroxides [nmol/ml]	3.67 ± 0.16	5.42 ± 0.18	< 0.001
III)	Antioxidants			
a)	Erythrocytic SOD [U/gm Hb]	1073 ± 58.2	1133 ± 51	< 0.05
b)	Serum Vitamin E [mg%]	0.930 ± 0.092	0.706 ± 0.065	< 0.001
c)	Serum Iron (μg%)	96.3 ± 3.45	81.93 ± 3.17	< 0.05

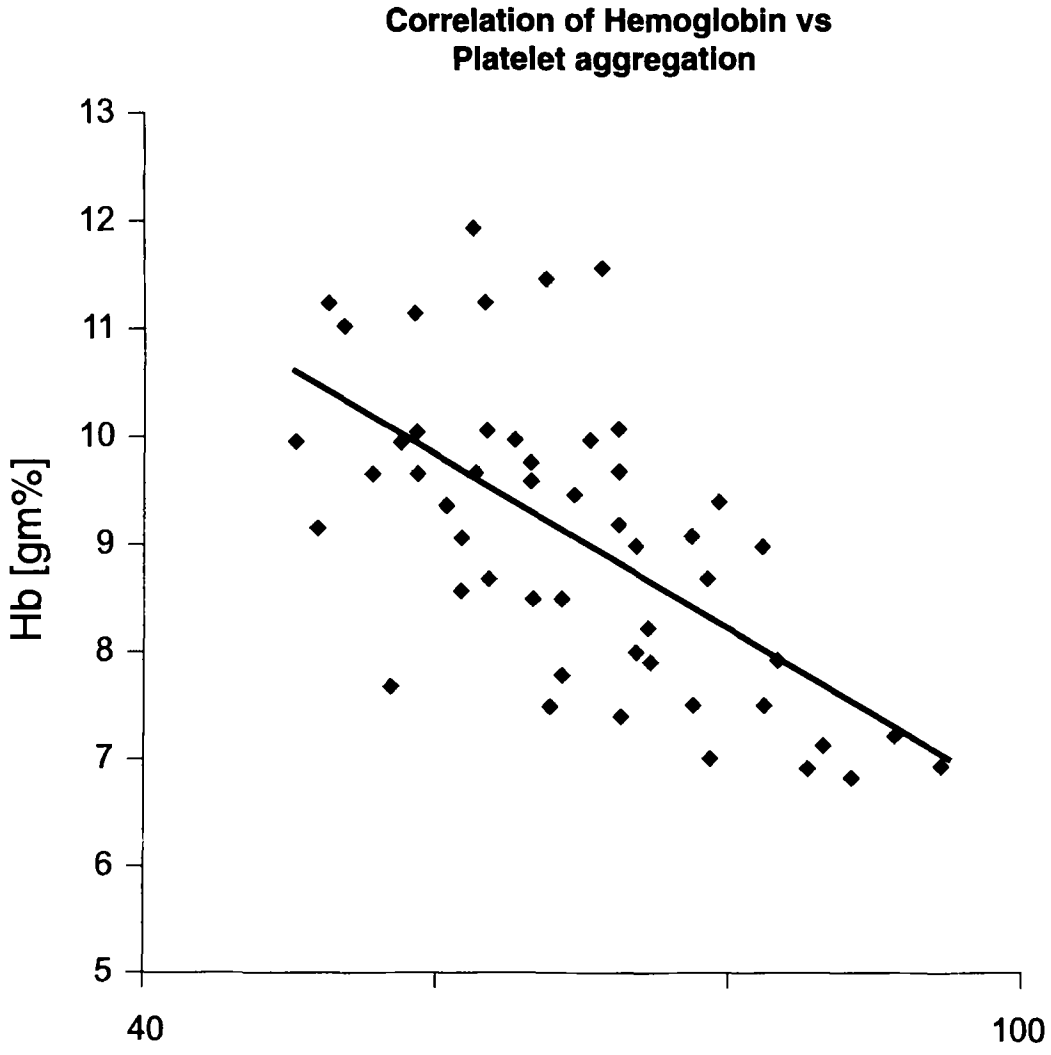
Values are expressed as Mean ± S.D. n = number of subjects studied.

Figure -1



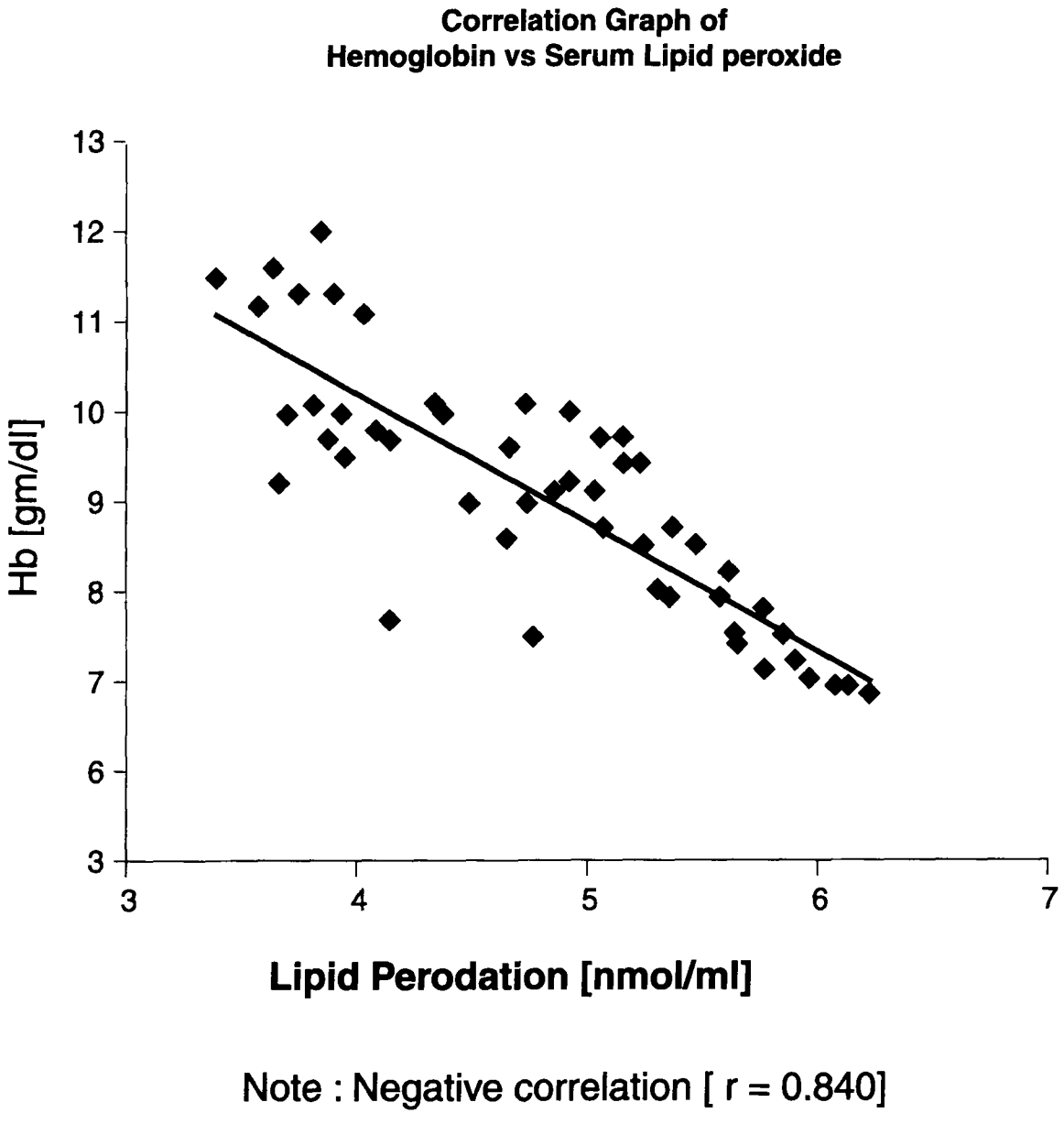
Note : Negative correlation [r = 0.720]

Figure -2



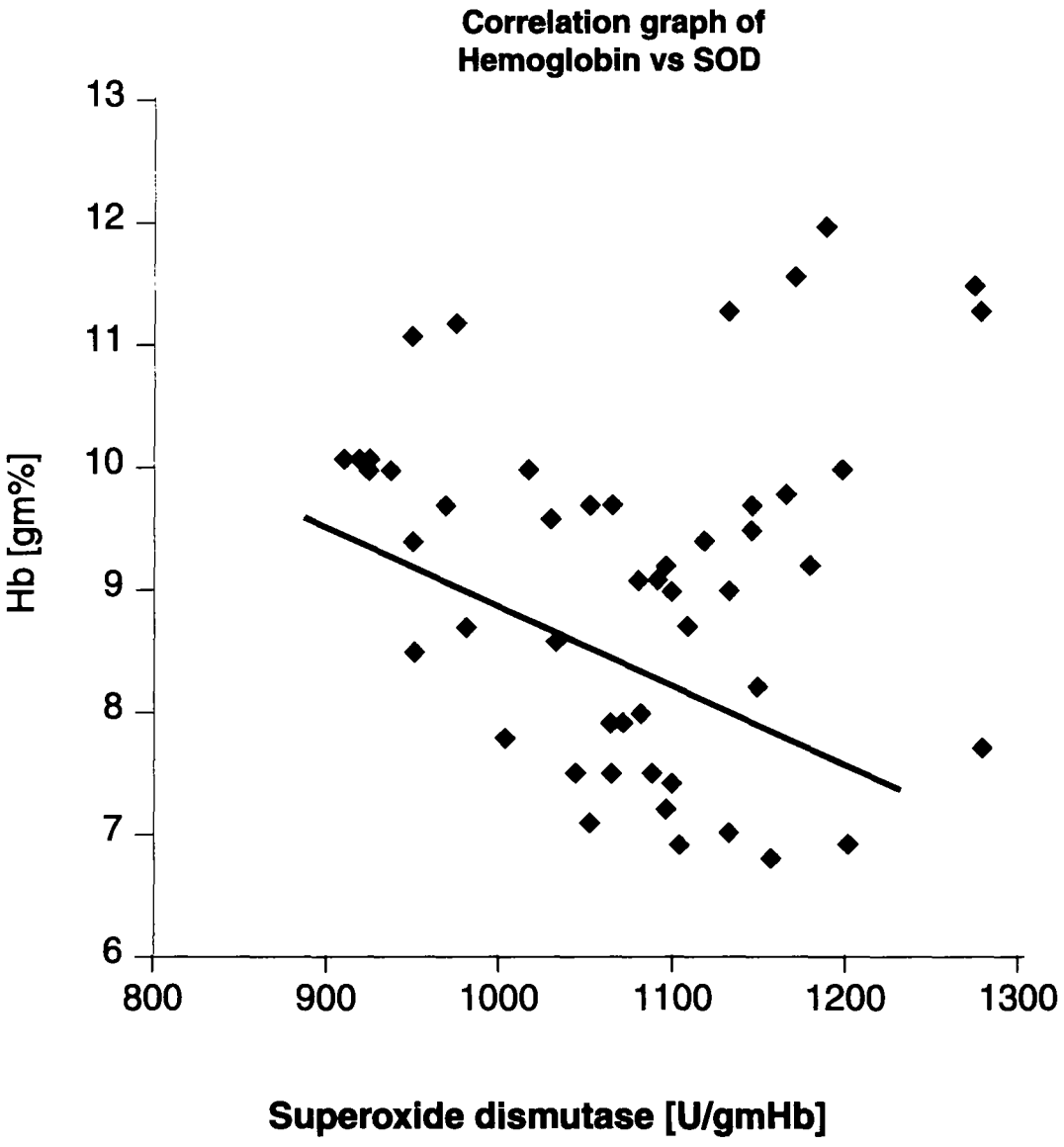
Note : Negative correlation [r = 0.637]

Figure -3



Note : Negative correlation [r = 0.840]

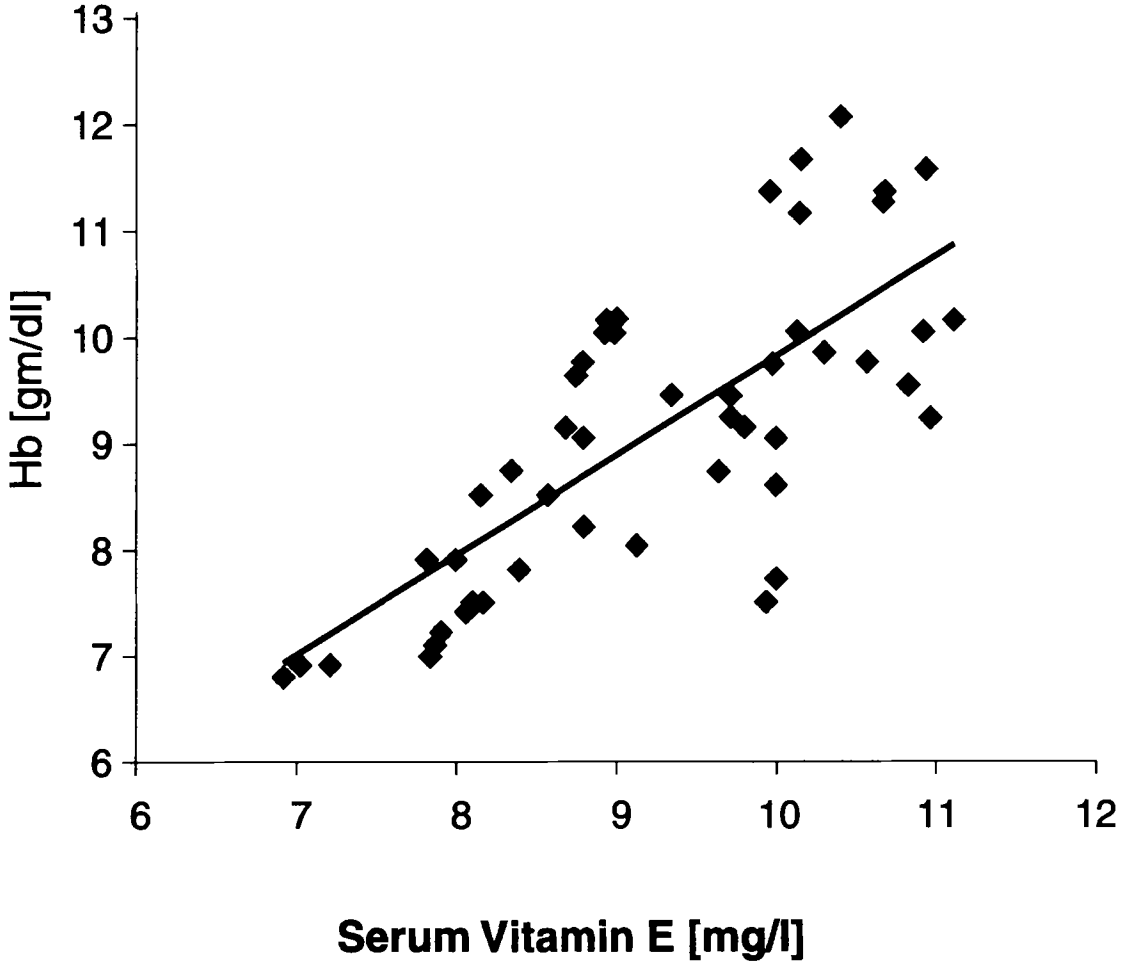
Figure -4



Note : Negative correlation [r = -0.504]

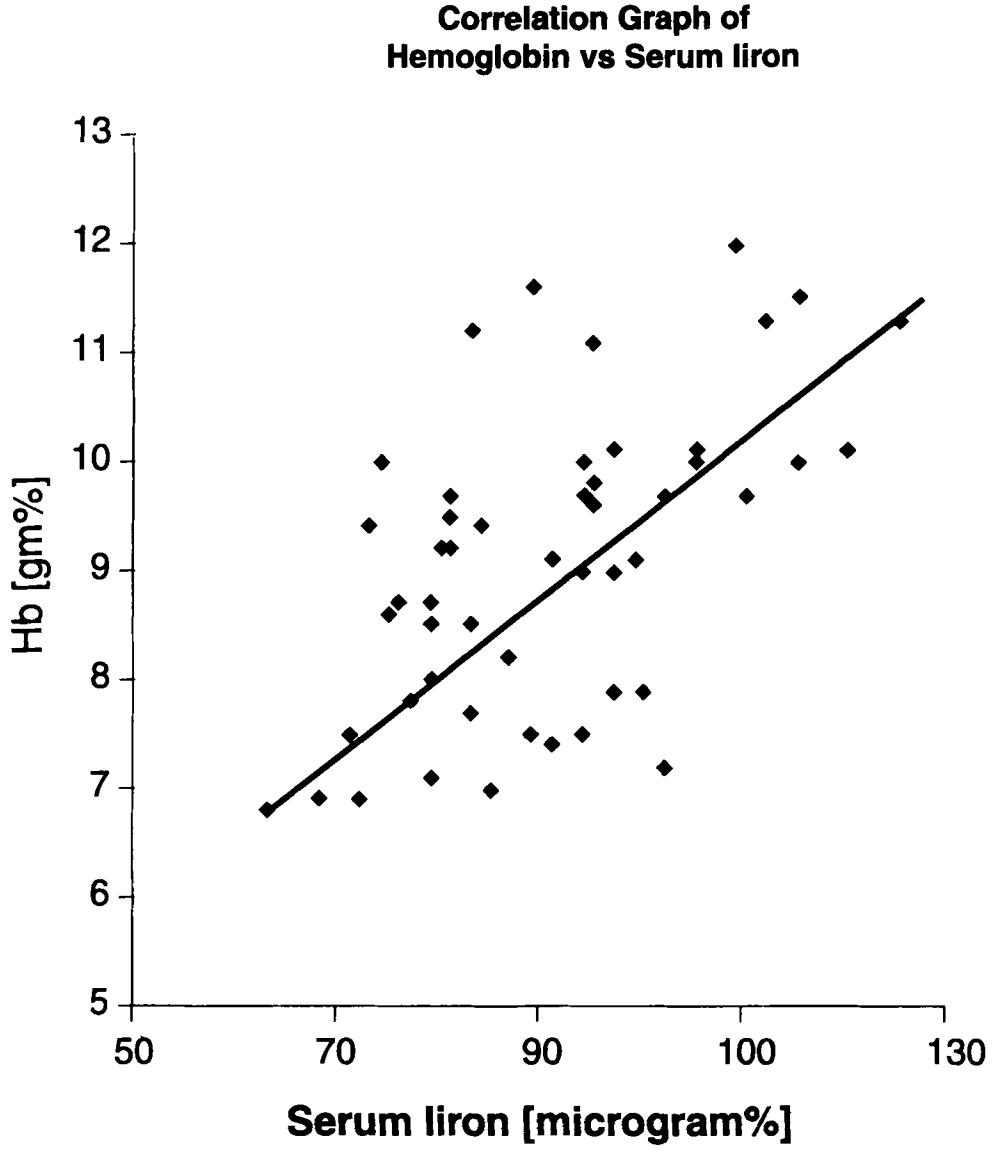
Figure -5

Correlation Graph of Hemoglobin vs Vitamin E



Note : Positive correlation [r = 0.739]

Figure -6



Note : Positive correlation [r = 0.571]

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