Antioxidant Enzymes and Oxidative Stress in the Erythrocytes of Iron Deficiency Anemic Patients Supplemented with Vitamins

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ABSTRACT

Background: Iron deficiency anemia is one of the major causes of morbidity and mortality worldwide. Evidences from epidemiological and clinical studies suggest a possible correlation between antioxidant levels and the anemic disease risk. The present work is to investigate antioxidant levels and lipid peroxidation in anemic patients. **Methods**: A number of 30 patients (15 males and 15 females) were selected for the study. Likewise, 30 age- and gender-matched healthy volunteers (15 males and 15 females) were selected with their informed consent. Patients and healthy subjects were supplemented with vitamins C and E for 15 days. The lipid peroxidation both in plasma and erythrocyte lysates was determined by thiobarbituric acid reactive substances and lipid peroxides. The antioxidant vitamins A, C, and E and total antioxidant activity were also analyzed. The antioxidant enzyme superoxide dismutase, catalase, and glutathione peroxidase were also determined. **Results:** Based on analysis, we found that the increase in lipid peroxidation was higher in the anemic subjects before vitamin supplementation, which was statistically significant at $P<0.05$. The antioxidant enzymes were higher in the patients before antioxidant supplementation when compared with patients after vitamin supplementation. **Conclusion**: Our data revealed higher oxidative stress before vitamin supplementation in iron deficiency anemic patients and after supplementation, lower lipid peroxidation and increased antioxidant vitamins were achieved. *Iran. Biomed. J. 18 (2): 82-87, 2014*

INTRODUCTION

Iron deficiency anemia (IDA) is one of the most common causes of morbidity and mortality worldwide, affecting people of all ages in both developed and developing countries. Oxidative stress is known to be a positive contributor for anemia, giving its effects on lipid peroxidation and DNA damage. Reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radicals are produced during aerobic metabolism [1]. Reactive oxygen species if not removed in a timely manner by an antioxidant system, mammalian cells may encounter oxidative stress that causes destruction of macromolecules and abnormal functions (neurotransmission function and altered immunologic and inflammatory defenses). Iron-mediated oxidative damage has been demonstrated *in vivo* in normal blood cells [2]. The constant auto-oxidation of hemoglobin generates superoxide radicals, which through spontaneous or enzymatic dismutation yields hydrogen peroxide [1, 2].

There are two main approaches to the use of supplements. They can be used to treat or prevent nutritional deficiency and to reduce the risk of non-deficiency disease and promote optimal health. The chemical versatility of iron has made it one of the most commonly used metals in biological systems. The iron deficiency continues to be a widespread condition affecting millions of people throughout the world [3]. In mammals, multiple physiological processes, including oxygen transport, respiration, DNA synthesis, formation of some neurotransmitters and hormones, xenobiotic metabolism, and certain aspects of host defense use iron-containing proteins [3-8].

The present study was designed to measure the oxidative stress parameters in iron deficiency anemic individuals expressed through plasma thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) before vitamins C and E supplementation and also to find out the changes in lipid peroxidation and antioxidant parameters after supplementation.

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MATERIALS AND METHODS

The study group included individuals who divided into two groups: IDA patients (15 male and 15 female) and healthy controls (15 male and 15 female). Healthy controls were selected from Bangalore University staff and students, while anemic individuals were selected from K.C. General Hospital, Malleswaram, Bangalore, India. The healthy individuals and anemic patients were evaluated before and after vitamin supplementation. Height and weight were measured to determine respective body mass index (BMI, kg/m^2) and surface area. The age ranged from 20 to 50 years. Criteria for IDA were hemoglobin concentration <11.5 g/dl in women and <13 g/dl in men, plasma iron concentration <45 μg/dl, and total iron-binding capacity >60 μmol/l.

Vitamins C and E (400 mg once daily) was supplemented to anemic as well as healthy controls for 15 days. Blood (5 ml) from normal and anemic individuals was collected before and after vitamin supplementation in a sterile vial containing ethylene diamine tetraacetate. Plasma was collected by centrifugation at 1,500 ×g for 15 min. The red blood cells (RBC) pellet was washed three times with chilled saline and lysed with chilled distilled water in a ratio of 1:4. The lysed RBC was centrifuged to obtain clear RBC hemolysate from cell debris. The hemoglobin level in whole blood was determined by Cyanmethemoglobin method using Beacon Diagnostics kit (Kabilpore, Navasari, India). Iron and total iron-binding capacity were determined by Ferrozine method using Coral Clinical Systems kit, Goa, India.

The lipid peroxidation product, malondialdehyde in plasma, was determined according to Buege and Aust [9] and LOOH was determined according to Jiang *et al.* [10]. CAT activity in hemolyzed RBC lysates was assayed in the RBC lysates by the method of Sinha [11], and SOD activity in hemolyzed RBC was determined by the method of Kakkar *et al.* [12], GPx was analyzed in RBC lysates by the method of Rotruck and co-workers [13] and glutathione content by the method of Beutler *et al.* [14]. Vitamin A was estimated by the methods of Bessey *et al.* [15], vitamin C (Ascorbic acid) by the method of Natelson [16] using dinitrophenyl hydrazine, and vitamin E by the method of Baker and Frank [17]. Total antioxidant activity was determined according to Benzie and Strain's method [18]. For statistical analysis, the packaged program SPSS (Statistical package for social sciences) for windows (version 13.0, Chicago, IL. USA) was used.

RESULTS

The biochemical parameters of iron deficiency anemic patients and healthy controls were determined before and after vitamin supplementation (Table 1). IDA patients were divided into male and female groups with respect to vitamin supplementation i.e., before and after vitamin supplementation. BMI both in cases and controls was in normal range, whereas the hemoglobin levels were lower in anemic group as compared to controls. The female patients showed lower hemoglobin levels as compared to male patients. However, after vitamin supplementation, a slight increase in hemoglobin levels was noticed among cases (both male and female group).

The oxidant parameters including TBARS and LOOH were increased before supplementation in cases as compared with controls, but their levels were found to be lowered after vitamin supplementation both in male and female cases. The enzymatic antioxidant parameter, CAT, was increased before vitamin supplementation; however, its level decreased after vitamin supplementation. Similarly, SOD and GPx levels were increased after vitamin supplementation.

The non-enzymatic antioxidant vitamins (A, C, and E) and total antioxidant activity showed increased levels after vitamin supplementation as compared to before vitamin supplementation. However, the levels of non-enzymatic antioxidants were lower than those of healthy controls. Male patients showed slight increased antioxidants as compared to female patients.

Based on independent *t*-test, BMI did not show any significance. Hemoglobin showed a highly significant relation when total case before supplementation was compared to after supplementation. Likewise, highly significant relation was obtained in male cases when before and after vitamin supplementation was compared. However, female cases did not indicate any significance.

The lipid peroxidation parameters did not show any significance except LOOH in females when before and after vitamin supplementation were compared. Total case, male, and female showed a significant relation of enzymatic antioxidants SOD and GPx when before vitamin supplementation group were compared with after vitamin supplementation group. However, CAT in male cases indicated a significant correlation when before and after vitamin supplementations were compared.

The independent *t*-test was carried out to find the significance between case and control which showed a highly significant (*P*<0.005) in all the oxidants, enzymatic, and non-enzymatic antioxidant parameters. The anemic case and control were compared before and after vitamin supplementation except CAT and SOD after vitamin supplementation. However, gender-wise comparison i.e., males to females before and after vitamin supplementation did not show any statistical significance.

Table 1. Biochemical parameters of anemic patients and controls before and after Vitamin Supplementation

Tests	Suppl	Total	Sig.	Anemic patients				Control
				Male	Sig.	Female	Sig.	
BMI	before	21.00 ± 0.38	0.651	20.45 ± 1.03	0.374	21.36 ± 1.70	0.628	20.48 ± 1.07[*]
	after	21.61 ± 0.08		20.18 ± 1.20		22.49 ± 1.98		20.68 ± 1.57[*]
Hb	before	9.21 ± 0.04	*0.000*	9.96 ± 0.19	*0.000*	8.46 ± 0.09	0.576	14.71 ± 0.83[*]
	after	10.53 ± 0.02		11.79 ± 0.16		9.27 ± 0.88		15.47 ± 0.63[*]
Lipid peroxidation parameters:								
TBARS	before	6.10 ± 0.42	0.150	6.39 ± 0.10	0.139	6.14 ± 0.79	0.482	3.21 ± 1.46[*]
	after	4.63 ± 0.73		5.14 ± 2.49		5.43 ± 0.62		3.54 ± 2.42[*]
LOOH	before	3.42 ± 0.82	0.466	3.12 ± 0.20	0.875	7.18 ± 0.02	*0.000*	2.51 ± 1.46[*]
	after	3.31 ± 0.70		2.99 ± 0.21		11.86 ± 0.95		2.24 ± 2.42[*]
Enzymatic antioxidant parameters:								
CAT	before	833.24 ± 55.27	0.549	855.66 ± 58.07	*0.034*	818.29 ± 53.57	0.331	358.62 ± 19.34[*]
	after	676.06 ± 56.08		565.17 ± 34.66		744.02 ± 65.93		180.88 ± 26.05
SOD	before	48.14 ± 1.62	*0.020*	46.04 ± 1.74	*0.000*	49.55 ± 1.18	*0.000*	58.41 ± 9.07[*]
	after	23.51 ± 0.52		23.14 ± 2.53		23.74 ± 1.65		64.75 ± 5.14
GPx	before	1.30 ± 0.07	*0.000*	1.20 ± 1.83	*0.001*	1.37 ± 0.25	*0.000*	3.85 ± 1.32[*]
	after	3.68 ± 0.17		3.55 ± 2.08		3.77 ± 0.26		4.21 ± 0.43[*]
Non-enzymatic antioxidant parameters:								
VITA	before	56.88 ± 0.83	0.262	60.04 ± 1.64	0.057	54.78 ± 6.89	0.544	75.53 ± 15.65[*]
	after	55.92 ± 0.90		58.72 ± 1.31		54.20 ± 7.60		82.26 ± 19.63[*]
VITC	before	0.44 ± 0.03	*0.015*	0.53 ± 0.07	0.347	0.38 ± 0.03	*0.009*	0.48 ± 0.26[*]
	after	0.55 ± 0.09		0.56 ± 0.03		0.54 ± 0.06		0.74 ± 0.25[*]
VITE	before	0.52 ± 0.01	0.455	0.51 ± 0.13	0.431	0.52 ± 0.12	0.333	1.12 ± 0.22[*]
	after	0.51 ± 0.017		0.55 ± 0.14		0.49 ± 0.14		1.22 ± 0.34[*]
TAA	before	517.00 ± 11.89	*0.009*	533.34 ± 16.03	0.105	506.11 ± 8.17	*0.025*	675.17 ± 12.41[*]
	after	581.43 ± 20.66		607.42 ± 22.22		565.50 ± 18.13		852.22 ± 14.36[*]

Italics and * denote significance; case versus control before and after vitamin supplementation (*P*<0.005). Suppl, vitamin supplemented group; BMI, body mass index; Hb, hemoglobin; TBARS, thiobarbituric acid reactive substances; LOOH, lipid hydroperoxide; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; VIT, vitmain; TAA, total antioxidant activity.

DISCUSSION

IDA is one of the most frequent causes of medical visits because of its high incidence in children, young women, and elderly people, especially if malnutrition is present. It is also one of the leading sings in many diseases or is the first evidence of disease observed in routine blood cell enumeration. In pathological state, an increase in the reactive free radicals creates an imbalance in this ration, thereby making macromolecules vulnerable to oxidative damage. The mammalian erythrocyte functions as an oxygen transporter, thus it is permanently in danger of oxidative damage. In order to avert this danger, the erythrocyte is well equipped with antioxidants and antioxidative enzymes [3, 4, 8, 19-21].

Lipid peroxidation has been accepted as being involved in the pathogenesis of various disorders and diseases [22]. Its inhibition is one of the important roles for antioxidants, as it induces disruption of membrane organization causing changes in fluidity and permeability, inhibition of metabolic processes, and alteration of ion transport. Furthermore, LOOH and their secondary reaction products, such as aldehydes, are cytotoxic, pro-inflammatory, and capable of modifying proteins and DNA bases [22]. Kumerova *et al.* [23] found that the antioxidant defenses were decreased, and the lipid peroxidation was increased in IDA patients. Tekin *et al.* [24] discovered that there was no significant difference in SOD and CAT activities in IDA patients relative to controls. In this study, we measured the oxidant and antioxidant status in IDA patients with age- and gender-matched healthy controls before and after vitamin supplementation. As a result of oxygen carrying function, erythrocytes accumulate oxidative damage during their life span in circulation. Protein aggregation, enzyme degradation, and lipid peroxidation have been shown to accompany

red cell aging and are accelerated in conditions such as diabetes and anemia [22]. The impairment of antioxidant defenses and the increase of oxidized lipids or proteins or susceptibility to oxidative stress are well known [23-26].

Isler *et al.* [27] reported that iron supplementation was used to recover the antioxidant defense system. Also, Bartal *et al.* [28] showed that although IDA cells are more susceptible to oxidation, they maintain good capacity for recovery. Kurtoglu *et al.* [29] demonstrated that oxidative stress was decreased after 6 week of iron supplementation, and the condition remained without significant changes until the end of the study, suggesting that oxidant-antioxidant status is regulated at the same time as hemoglobin normalization. They also found that decreased lipid peroxidation, which was expressed as lower malondialdehyde levels in both groups of IDA patients receiving iron supplements and oxidative stress, was lower both at the time of hemoglobin normalization and at the time of saturation of iron body stores.

Nagababu *et al.* [30] reported that severe anemic RBC undergoes more oxidative stress than normal cells. The damage to the red cell explains that the shorter red cell life-span associated with anemia. Troost *et al.* [31] study showed that iron ingestion *in vivo* induces intestinal oxidative damage as indicated by the occurrence of lipid peroxidation. The magnitude of the TBARS concentration in the intestinal fluid samples as a result of iron ingestion was remarkably high. TBARS concentration in the intestinal fluid samples after the iron challenge was roughly 3-5 times higher than those observed in plasma in healthy adults. Our findings were in agreement with those of Bartal *et al.* [28], Kuypers *et al.* [32], Chiu and Kuypers [33], and Amirkhizi *et al.* [34] that reported erythrocytes in IDA were more susceptible to oxidation, but had good capacity for recovery. They also reported a significant decrease in TBARS levels in anemic patients after iron supplementation with ferrous sulfate [32-34]. Although erythrocytes possess highly active antioxidant enzymes, such as SOD, GPx, and CAT compared to other cell types, our results showed that females had slightly increased SOD levels before and after vitamin supplementation as compared to males. Interestingly, GPx also showed increased levels after vitamin supplementation in females as compared to males. CAT showed decreased levels after vitamin supplementation in both male and female anemic groups.

A treated group of nine IDA patients with iron, containing drugs, vitamins, and folic acid in the presence of either vitamin C (500 mg) or combination of Geritamin (30,000 UL vitamin A and 0.07 g vitamin E) plus vitamin C (0.5 g) daily for 3 months, explaining that after therapy with iron in IDA patients,

only the level of plasma ceruloplasmin was normalized. Malondialdehyde was found to remain high, the activities of SOD and GPx remained lower, and CAT was found to remain very high as before iron treatment. Our study illustrated that repletion of IDA patients with iron promoted oxidative stress. The causative factor responsible for such effects was hydroxyl radical produced by excess iron. Iron treatment may give rise to increased free iron concentration in cells, which may induce free radical production. In IDA patients treated with iron along with the antioxidant vitamins A, E, and ascorbic acid, the oxidative stress was reduced and the activity of SOD was normalized [35].

Antioxidant vitamins and trace elements contribute to maintain an effective immune response. Maintaining adequate antioxidant status may provide a useful approach in attenuating cell injury and dysfunction. Beneficial effects of oral supplementation of vitamin E (500 mg/day for 6 months) are improvement of anemia correction, atherosclerosis prevention, and increase in osmotic resistance of RBC. A significant decrease in TBARS and increase in nitric oxide, vitamin E, vitamin C, CAT, and SOD were observed on supplementation of vitamin E (400 IU/day for 2 months), which reflected a proper absorption of vitamin by the body [33-35].

By contrast, on supplementation of 100 mg iron form (fumarate) and 500 mg vitamin C to pregnant women showed increased lipid peroxidation and decreased vitamin E level. The potential harmful effects of a high iron supplementation during pregnancy were determined, and deleterious effect of the combined iron/vitamin C supply could be related to its prooxidant properties. Iron deficiency is regarded as the major cause of nutritional anemia, insufficient levels of vitamins B_{12}, A, C, and E folate, and riboflavin has been found to be associated with this deficiency. Some clinical trials suggested that vitamin E might have a role as a potential erythropoietic agent in the anemic patients suffering from various types of inherited hemolytic anemia, chronic renal failure patients on hemodialysis, and mildly anemic apparently healthy subjects with borderline anemia. Vitamin E has also the potential to be effectively used for preventing and/or treating some types of human anemia due to its putative role in promoting erythropoiesis, enhancing the integrity and stability of erythrocyte membrane proteins and lipids, and reducing the oxidative stress-induced erythrocyte fragility and lysis resulting in increased RBC survival and hemoglobin level [27, 30-35].

In conclusion, we found that oxidant status was increased in IDA patients; however, it was slightly decreased after supplementation of vitamins C and E. The non-enzymatic antioxidants were also increased

after vitamin supplementation in anemic patients. Our results suggested that peroxidative damage would be prevented in patients on supplementation of vitamins. Future studies are being carried out in our laboratory based on a larger number of samples and iron supplementation to anemic patients.

ACKNOWLEDGEMENTS

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