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Original Article

Assessment of lipid peroxidation and activities of erythrocyte cytoprotective enzymes in women with iron deficiency anemia

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Abstract

BACKGROUND: The aim of this study was to investigate whether the lipid peroxidation levels and activities of erythrocyte cytoprotective enzymes can be affected by iron deficiency anemia (IDA) in menstruating women.

METHODS: In this case-control study, 43 women with IDA, 43 women with iron deficiency (ID) and 43 healthy controls were included. Lipid peroxidation levels were assessed by measuring the concentrations of plasma malondialdehyde (MDA) (as byproduct of lipid peroxidation). We also evaluated the activities of erythrocyte cytoprotective enzymes by measuring activities of copper zinc-superoxide dismutase (CuZn-SOD), glutathione peroxidase (GPX) and catalase (CAT) in selected groups. Furthermore, total antioxidant capacity (TAC) of plasma was assessed for each subject. Comparisons between continuous variables across groups were performed by the calculation of one-way analysis of variance (ANOVA).

RESULTS: Mean plasma concentration of MDA was significantly higher in IDA group than that in ID group (3.49 ± 0.84 vs. 2.76 ± 0.59, µmol/L, respectively, $p < 0.01$) and healthy group women (3.49 ± 0.84 vs. 2.94 ± 0.71 µmol/L, respectively, $p < 0.01$). No significant difference was observed between ID and healthy groups in plasma MDA concentration (2.76 ± 0.59 vs. 2.94 ± 0.71, respectively). The mean erythrocyte CuZn-SOD activity in IDA group was significantly lower than that in healthy group women (674 ± 89 vs. 796 ± 82, respectively, $p < 0.01$). Furthermore, erythrocyte CAT activity was significantly lower in IDA group compared to both ID (162 ± 52 vs. 193 ± 72, respectively, $p < 0.01$) and healthy women groups (162 ± 52 vs. 234 ± 68, respectively, $p < 0.001$). No significant difference was observed between study groups in erythrocyte GPX activity. In addition, plasma TAC levels were significantly lower in IDA and ID groups compared to healthy women group (1.97 ± 0.42 and 2.16 ± 0.64 vs. 3.76 ± 0.86, respectively, $p < 0.01$).

CONCLUSIONS: Our findings showed that activities of erythrocyte cytoprotective enzymes decrease and lipid peroxidation increases in women with IDA. The consequence of the low activity of the cytoprotective enzymes in human is progressive tissue damage, which may eventually lead to atherosclerosis and other degenerative diseases.

KEYWORDS: Lipid peroxidation, iron deficiency anemia, cytoprotective enzymes, women.

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Iron deficiency anemia (IDA) is the most common nutritional disorder worldwide, affecting people of all ages in both industrialized and developing countries.[1] Iron-mediated oxidative damage has been demonstrated in vivo in normal red blood cells

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(RBC).[2] The constant auto-oxidation of hemoglobin generates superoxide radicals, which through spontaneous or enzymatic dismutation yield hydrogen peroxide. The involvement of free and hemoglobin-associated iron species in the generation of highly reactive radicals has recently been reviewed.[3] Furthermore, previous studies have also reported a positive association between serum iron levels and lipid peroxidation.[4] Based on the nature of iron toxicity,[5] iron deficiency would not be expected to give increased lipid peroxidation, unless a decrease in the iron dependent enzymes impairs the antioxidant mechanisms of RBC. It is therefore unclear whether the ability of RBC to undergo lipid peroxidation is increased or decreased during IDA in menstruating women. Impairment of the antioxidant defense system and reduced cellular immunity and myeloperoxidase activity were previously reported in patients with IDA.[6,7] All of these may contribute to inadequate erythrocyte survival. The literature offers contradictory and limited data on oxidative stress and antioxidant defense in patients with IDA.[8,9] Determination of antioxidative capacity is now considered as a tool in medical diagnosis and treatment of several diseases, including cardiovascular diseases, cancers, diabetes mellitus and aging.[10] Total antioxidant capacity (TAC) considers the cumulative action of all the antioxidants present in plasma and body fluids and provides an integrated parameter rather than the simple sum of measurable antioxidants. There is now a wide rang of evidence indicating the importance of TAC in plasma and modification during oxidative stress development, as well as its feasibility as a tool for investigating the association between diet and oxidative stress.[11] The objectives of the present study were: (1) to determine whether lipid peroxidation was increased in IDA; if so (2) to determine the activities of erythrocyte cytoprotective enzymes and plasma TAC levels. Thus, lipid peroxidation was assessed by measuring the concentrations of plasma malondialdehyde (MDA). We also measured activities of erythrocyte copper zinc-superoxide dismutase (CuZn-SOD), glutathione peroxidase (GPX) and catalase (CAT) and plasma TAC levels in selected groups of women with IDA.

Methods

The subjects used in this study were recruited from menstruating women (20-45 year old) under the cover of rural health centers of Kerman province, Iran. Pregnant and lactating women and subjects with history of cancer, cardiovascular disease, diabetes and renal or liver diseases and those taking vitamin or mineral supplements were excluded. Informed written consent was obtained from subjects before entering the study. Data collecting form included demographic characteristics such as age, number of pregnancies, physical activity and detailed medical history. Nutritional habits was assessed using a validated food-frequency questionnaire, extensively described elsewhere. Physical activity was classified as active if subjects reported "moving" walking and working energetically and participating in vigorous exercise, otherwise, they were classified as inactive. Body weight was measured while the subjects were wearing light clothing without shoes to the nearest 0.1 Kg. Height was measured to the nearest 1 cm while subjects were not wearing shoes, in standing position. Body mass index (BMI) was calculated as weight (in kilogram) divided by height (in meters-squared). To calculate waist-to-hip ratio (WHR), the waist circumference was measured in a horizontal plane at the level of the high point of iliac crest to the nearest 0.1 cm. Hip circumference was measured in a horizontal plane at the maximum extension of the buttocks. Venus blood samples were obtained from median cubital vein and collected into standard tubes containing ethylene diamine tetra acetic acid (EDTA). Blood samples centrifuged at 3000 rpm for 10 minutes at 4°C and plasma was separated for the assay MDA. The buffy coat was removed and remaining erythrocytes were washed three times in cold saline (9 g/l NaCl) and hemolysed by the addition of

an equal volume of ice-cold demineralized ultrapure water. Subjects' plasma and hemolysates were stored in -70°C until analysis.

Plasma ferritin concentrations were determined with Radioimmunoassay method and standard kits (Spectria kit, Orion Diagnostica, Fenland). Blood hemoglobin and mean corpuscular volume (MCV) were measured by Sysmax A-380 automated cell counter. Description criteria for IDA are microcytic hypochromic erythrocytes with a mean corpuscular volume < 80 fL, hemoglobin concentration ≤ 12 g/dL and plasma ferritin concentrations < 15 µg/L while iron deficiency without anemia (ID) is defined as hemoglobin concentration > 12 g/dL and plasma ferritin < 15 µg/L.[12] Forty-three women were recruited as an IDA group and 43 women were selected as ID group. Forty-three age-matched healthy women were also recruited as control group. Plasma MDA concentrations were assayed by measurement of thiobarbituric acid reactive substances according to Satoh method.[13] MDA produced by hydrolysis of lipid hydroperoxides when heated under acid conditions reacts with thiobarbituric acid (TBA) to form a red complex, which absorbs light maximally at 530 nm. The complex is usually measured after extraction into butanol (to improve sensitivity) and is quantified against MDA standards generated from tetramethoxypropane under the same reaction conditions. The method used in this study is based on linking two molecules of TBA with a molecule of MDA at 95°C in an acid environment. In order to express the activities enzymes per gram hemoglobin (Hb), Hb concentration was measured in the hemolysates with a standard kit (Zist chemistry Laboratories, Iran) involving the cyanmethemoglobin method (Drabkin's method). Catalase activity was determined by method of Hygo Aebi.[14] Activity of CAT was determined by following the decomposition of H_2O_2 in phosphate buffer pH 7.2, spectrophotometrically at 230 nm. One unit of CAT is defined as the amount of enzyme, which liberates half the peroxide oxygen from a H_2O_2 solution in 100 second at 25°C. Enzyme activity was expressed as units per g of Hb (U/g Hb). Glutathione peroxidase (GPX) activity was measured according to Paglia and Valentine method.[15] GPX converts the reduced glutathione (GSH) to oxidized glutathione (GSSG). In the presence of glutathione reductase and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. One unit of GPX was defined as the amount of enzyme that catalyzed the transformation of 1 µmol of NADPH per minute under the assay condition. The activity of GPX in units/l divided by Hb concentration in g/l gives the activity of GPX in units/g Hb.

Measurement of copper zinc-superoxide dismutase (CuZn-SOD) activity was performed by kit Ransod (Randox Laboratories, cat. no. SD-125). The role of CuZn-SOD is to accelerate the dismutation of the toxic superoxide radical to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The CuZn-SOD activity is then measured by the degree of inhibition of this reaction. One unit of CuZn-SOD is that which causes a 50% inhibition of the rate of reduction of INT under the condition of the assay. Enzyme activity was expressed as CuZn-SOD units/g Hb DATA analyses. Plasma TAC levels were determined by colorimetric assay using 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS).[16] The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS to $ABTS^{\bullet+}$ by a peroxidase. The amount of produced $ABTS^{\bullet+}$ can be monitored by reading the absorbance at 600 nm. The coefficients of repeatability, expressed as a percentage of the nearly maximal variation, namely 4 s.d. of the measurement under investigation, ranged from 2 to 7% for SOD, GPX and CAT. The coefficient of repeatability for MDA was 4%. Inter-assay variability was calculated as reproducibility from day to day for the laboratory pa-

rameters and expressed as the relative SD derived from the mean value. Inter-assay CV_s ranged from 4 to 8%. For each variable, values are expressed as mean ± standard deviation (SD). Normality tests were applied using the Kolmogorov-Smirnov criterion. Associations between categorical variables were tested by the use of chi-squared test. The differences between groups were sought by using one-way analysis of variance (ANOVA) for continuous variables. Linear relationships among dependent (MDA, GPX, CuZn-SOD and Catalase) and independent (iron intake, Hb, Plasma iron, ferritin) variables were evaluated by Pearson's correlation coefficients. Two-tailed values of $p < 0.05$ were considered statistically significant. SPSS version 12.5 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) software was used for all statistical calculations. For all significant tests, a power of $(1-\beta) \geq 0.8$ with $\alpha \leq 0.05$ was achieved.

Results

Demographic, anthropometric and nutrient intake characteristics of the participants are shown in table 1. Mean of age, weight, BMI, WHR, number of pregnancies and selected nutrient intakes were similar in study groups (table 1). Table 2 shows that plasma concentration of MDA was significantly higher in IDA group than that in ID and healthy group women ($p < 0.01$). No significant difference was observed between IDA and ID groups in plasma MDA concentration. The mean erythrocyte CuZn-SOD activity in IDA group was significantly lower than that in healthy group women ($p < 0.01$). Furthermore, erythrocyte CAT activity was significantly lower in IDA group compared to both ID ($p < 0.01$) and healthy women groups ($p < 0.001$). No significant difference was observed between three groups in erythrocyte GPX activity (table 2). In addition, plasma TAC levels were significantly lower in IDA and ID groups compared to healthy women group ($p < 0.01$). In the overall samples, plasma MDA levels were positively correlated with plasma iron levels ($r = 0.250$, $p < 0.001$). Furthermore, Hb concentration was positively correlated with erythrocyte CAT activity ($r = 0.246$, $p = 0.03$) and plasma TAC levels($r = 0.321$, $p = 0.005$). We found that plasma ferritin levels are positively correlated with erythrocyte CAT activity ($r = 0.258$, $p = 0.02$) and plasma TAC levels ($r = 0.436$, $p < 0.001$) (table 3).

Table 1. Demographic, anthropometric and nutrient intake characteristics of the study groups.*

Variables	IDA group (*n*=43)	ID group (*n*=43)	Healthy group (*n*=43)
Age (years)	27 ± 11	28 ± 7	29 ± 12
Weight (kg)	62.3 ± 6.4	64.4 ± 5.8	67.2 ± 7.2
Height (cm)	157.4 ± 5.9	159.3 ± 6.1	158.6 ± 5.3
Body mass index (kg/m^2)	24.4 ± 4.2	25.2 ± 3.7	25.6 ± 5.4
Waist-to hip ratio	0.72 ± 0.06	0.77 ± 0.05	0.78 ± 0.08
Physical inactivity (n)	37	39	36
Number of pregnancies	3.4 ± 2.1	3.5 ± 2.7	3.8 ± 3.1
Energy intake (kcal/d)	1664 ± 304	1715 ± 417	1728 ± 426
Protein (%energy)	12.4 ± 1.3	12.6 ± 1.8	13.1 ± 1.6
Total fat (%energy)	30.2 ± 4.0	32.7 ± 4.6	31.4 ± 5.2
Carbohydrate (%energy)	57.6 ± 5.2	54.7 ± 3.9	55.5 ± 5.1
Iron intake (mg/day)	17.8 ± 2.7	18.6 ± 3.6	19.2 ± 4.2

*mean ± SD

IDA: Iron deficiency anemia, **ID:** Iron deficiency without anemia

Table 2. Concentrations of plasma MDA and activities of erythrocyte CuZn-SOD, GPX and CAT in study groups.

Variables	IDA group (*n*=43)	ID group (*n*=43)	Healthy group (*n*=43)	p value‡
MDA (µmol/L)	3.46 ± 0.84*, †	2.76 ± 0.59	2.94 ± 0.71	0.004
CuZn-SOD (U/gHb)	674 ± 89*	719 ± 102	796 ± 82	0.002
GPX (U/gHb)	62.7 ± 14.8	64.3 ± 15.7	65.8 ± 14.3	0.324
CAT (K/gHb)	162 ± 52**, †	193 ± 72*	234 ± 68	0.001
TAC (µmol/L)	1.97 ± 0.42*	2.16 ± 0.64*	3.79 ± 0.86	0.006

‡ p values were calculated from the one-way ANOVA.
* $p < 0.01$ from the post hoc comparisons (Scheffe test) between IDA or ID subjects compared healthy subjects.
** $p < 0.001$ from the post hoc (Scheffe test) comparisons between IDA or ID group compared healthy subjects.
† $p < 0.01$ from the post hoc (Scheffe test) comparisons between IDA group compared ID group.

Table 3. Pearson's correlation coefficients between dependent and independent variables (N = 129).

Variables	MDA (µmol/L)		CAT (K/gHb)		GPX (U/gHb)		CuZn-SOD (U/gHb)		TAC (µmol/L)	
	r	p	R	p	r	P	r	p	r	p
Hb (g/dL)	0.041	NS*	0.246	0.03	-0.080	NS	0.042	NS	0.321	0.005
Plasma iron (µg/dL)	0.250	< 0.001	0.058	NS	-0.064	NS	-0.048	NS	0.029	NS
Plasma ferritin (µg/L)	0.032	NS	0.258	0.02	0.088	NS	-0.056	NS	0.436	< 0.001
Iron intake (mg/day)	0.074	NS	0.065	NS	0.042	NS	0.038	NS	0.112	NS

*NS: Not significant.

Discussion

Lipid peroxidation is a free radical-generating process, which occurs on every membranous structure of the cell. Free radicals are known to be involved in a number of human pathologies including atherosclerosis.[17] In the present study, we observed increased levels of plasma MDA (as byproduct of lipid peroxidation) in women with IDA, which may be attributed to over production of reactive oxygen species (ROS_s) or a deficiency of antioxidant defense. Although previous studies have suggested that IDA may be related to increased lipid peroxidation,[8,18-20] the exact mechanism by which increased lipid peroxidation in theses patients has not been completely clarified. Among them, Bartal et al[19] reported that erythrocytes in IDA were more susceptible to oxidation, but had good capacity for recovery, so that significant decrease in MDA levels in anemic patients after iron supplementation with ferrous sulfate has been reported.[8,20] Two possible factors may lead to increased lipid peroxidation: the increasing production of free radicals and the declining activity of the antioxidant system. Antioxidant enzymes are the major defense systems of cells in normal aerobic reactions.[21,22] Erythrocytes are equipped with a highly effective antioxidant defense system. After we confirmed a positive association between IDA status and plasma MDA levels, we attempted to identify factors associated with increased MDA levels. Therefore, we studied activities of erythrocyte antioxidant enzymes and plasma TAC levels in participants. Although, erythrocytes possess highly active antioxidant enzymes, such as CuZn-SOD and GPX compared to other cell types,[9] but our results showed that women with IDA have lower CuZn-SOD activity than ID and healthy women. These results were in line to previous reports which found decreased CuZn-SOD in IDA.[9,20] In contrast, some researchers observed increased SOD activity in patients with IDA.[6,23] In the present

study, SOD activity decreased in both IDA and ID groups but this was only significant in IDA group, probably because the iron-deficient changes were more marked in this group. Decreased CuZn-SOD activity in IDA may be linked to increased oxidant stress; because, it is well know that reactive oxygen species, especially hydrogen peroxide, inhibit SOD activity.[24] We also found the lower CAT activity in IDA and ID groups compared to controls. This finding, was in agreement, however, with those of Acharya et al[6] who found decreased activities of CAT in patients with IDA. CAT is an iron-dependent enzyme and is not unexpected to be decreased in iron deficiency. The positive relationship, which we observed between Hb and ferritin concentration with CAT activity was confirmed this finding. Interestingly, GPX activity in IDA group was similar to healthy control group. This finding comes in accordance with the findings of Isler et al[24] who showed that GPX activity in patients with iron deficiency anemia was similar to healthy control group. Inversely, Sevgi et al[25] in their study reported decreased GPX activity in children with ID. In contrast to our result, Yetgin et al[26] also found the positive correlation between GPX activity and serum iron levels. It has been reported that the activities of pentose phosphate pathway enzymes, the major source of the NADPH production, were increased in patients with IDA.[24] Since GPX activity depends on NADPH levels produced by pentose phosphate pathway, it was within the normal levels in women with IDA, as opposed to significantly low SOD activity in comparison with healthy women. However, these controversial findings may be partially due to differences in duration of the IDA, stage of anemia, laboratory methods used and/or sample size.

In the present study, the status of the plasma TAC levels was investigated in participants. We observed that plasma TAC levels were lower in IDA and ID women than that in healthy women. TAC considers the cumulative action of all the antioxidants present in plasma and body fluids and provides an integrated parameter rather than the simple sum of measurable antioxidants. To our knowledge, no previous study has investigated the plasma TAC levels in IDA women and therefore, we were not able to compare our data with other studies in populations. However, lower concentration of serum selenium as antioxidant mineral, has been previously reported.[26] The increase in plasma MDA levels in IDA women may be partially due to decline in plasma TAC levels. Our study may have some limitations in data gathering like all cross-sectional studies. First, as with all observational studies, our results could be biased by unrecognized confounders. Second, the cross-sectional study does not allow us to conclude causal relationships. Third, we were not able to assess nutrient intakes (including vitamins and minerals) of participants. Other limitations of our study were the limited number of subjects, and the fact that only women were included in the analysis. In conclusion, our data indicated that activities of erythrocyte cytoprotective enzymes decrease and oxidative stress increases in women with IDA. The consequence of the low activity of the cytoprotective enzymes in human is progressive tissue damage, which may eventually lead to atherosclerosis. These associations in later life may have important implications for the development of atherosclerosis and the long-term cardiovascular health of women, and warrant further investigations, particularly in those women at increased risk.

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