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Increased oxidative stress in adult women with iron deficiency anemia

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ABSTRACT

BACKGROUND
Iron deficiency anemia (IDA), a type of anemia with an increasing global frequency, is more common in women than men in the population. In IDA, the sensitivity of erythrocytes to oxidants is increased and their lifespan is shortened. Oxidative stress is an imbalance between free radicals and antioxidant molecules which is one of the potential biochemical mechanisms involved in the pathogenesis of IDA. In our study, we aimed to determine the levels of oxidant and antioxidant markers by assessing the levels of total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), paraoxonase-1 (PON-1), and myeloperoxidase (MPO) in women with IDA.

METHODS
This was a cross-sectional study involving 47 women with IDA aged ≥40 years and 47 women volunteers. The levels of TAS, TOS, OSI, PON-1, and MPO were determined spectrophotometrically using appropriate kits. Non-parametric Mann Whitney-U tests were used to analyze the data.

RESULTS
The levels of antioxidants TAS (1.42 mmol Trolox equiv./L) and MPO (54.00 U/L) in the IDA group were significantly lower than in the control group [TAS (1.67 mmol Trolox equiv./L) and MPO (89.00 U/L)] (p=0.000 and p= 0.019, respectively). However, TOS (6.25 μmol H_2O_2 equiv./L) level in the IDA group was significantly higher than in the control group (4.13 μmol H_2O_2 equiv./L) (p=0.000), but PON-1 was not significantly different between the two groups (p=0.375).

CONCLUSION
In women with IDA, the oxidant-antioxidant balance is impaired, resulting in oxidative stress. Therefore, IDA in adult women must receive adequate attention in clinical practice.

Keywords: Iron deficiency anemia, antioxidants, paraoxonase-1, myeloperoxidase, oxidative stress, women.

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INTRODUCTION

Anemia is called iron deficiency anemia (IDA) when the iron that the body loses daily cannot be compensated for by the iron taken with food. Iron deficiency is the most common cause of anemia in the world and is more common in women than men.[1,2] Despite the decline in the prevalence of anemia worldwide, the burden of anemia remains consistently high in many regions. Greater focus on interventions is needed to accelerate progress towards the Global Nutrition Target 2, which is to reduce anemia in women of reproductive age by 50% by 2025 and to alleviate significant health losses in children under five.[3] The total amount of iron in the body is 4-5 grams, of which about 65% is in hemoglobin, 4% in myoglobin, 1% in heme compounds that facilitate intracellular oxidation, and 0.1% is associated with transferrin protein in blood plasma. In addition, 15-30% of iron is stored as ferritin in the reticuloendothelial system and liver parenchymal cells.[4] A fine balance exists between dietary intake and loss of iron and is normally maintained in a healthy person. The iron loss is approximately 1 mg daily through the shedding of cells from mucosal and cutaneous surfaces, but in premenopausal female adults it may increase to about 2 mg through menstruation. In addition, iron requirements are temporarily greater during neonatal and childhood growth spurts because of increased body mass.[5,6]

With the formation of free radicals under normal physiological conditions, the antioxidant defense system is in a continuous state of alertness. Antioxidants retain reactive oxygen species (ROS) within normal limits and reduce their harmful effects.[7] If this equilibrium slips to oxidants, oxidative stress occurs, and it creates ROS and damages the body.[8] Reactive oxygen species plays a role in the pathogenesis of various diseases such as malignancies, systemic lupus erythematosus, coronary artery disease, rheumatic diseases, diabetes mellitus, and Bechet's disease.[9] Recently, studies were performed on oxidant/antioxidant molecules. It is possible to measure the plasma levels of the oxidizing molecules separately, but this may not be appropriate, as these molecules can affect each other. Therefore, total oxidant status (TOS) level reflecting the oxidant status is used.[10-11] The measurement of TOS alone is more enlightening than the individual measurements of the oxidants. For the same reason, instead of individual antioxidant measurements, total antioxidant status (TAS) measurements are more widely used.[10] Besides, the enzyme myeloperoxidase (MPO) is a lysosomal enzyme secreted from leukocytes in response to oxidative stress. Myeloperoxidase is a tetrameric, glycosylated, heme-containing protein.[12] Myeloperoxidase produces free radicals and oxidants with its antimicrobial effects, thus causing oxidative damage in the main tissues in regions of inflammation.[13,14] An antioxidant, paraoxonase-1 (PON-1), which is synthesized by the liver, is transported in plasma depending on *high-density lipoprotein* (*HDL*). It ensures the anti-atherogenic features of HDL, by avoiding low-density lipoprotein (LDL) oxidation.[15]

Iron deficiency is an important health problem of women over the age of 40, which is frequently encountered in our country as well as all over the world and creates a certain burden on the health economy of a country.

A cross-sectional study showed that mean paraoxonase-1 (PON-1) in the iron-deficiency anemia group was significantly lower than in the control group (102.4 ±19.2 U/L and 163.3 ±13.68 U/L; $p=0.000$).[16] A cross-sectional observational study among men and women with anemia and without anemia (i.e., healthy controls), showed that TAS levels did not vary significantly among the categories (p=009).[17] However, another study among 26 subjects with IDA and 20 healthy controls showed different results, the serum TAS being significantly lower in patients with iron deficiency anemia than in controls ($p<0.05$).[18] There were inconsistent results of the previous studies, therefore, a study

was performed to compare TAS between IDA and healthy controls. In our study, it was aimed to evaluate more than one oxidative stress parameter together in female patients diagnosed with iron deficiency anemia. In this study, we planned to compare a number of oxidative stress markers (TAS, TOS, serum MPO, and PON-1 levels) between patients with IDA and healthy controls.

METHODS

Research design

The present cross-sectional observational study was conducted at the Internal Medicine outpatient clinic between January and December 2020.

Research subjects

Healthy female individuals who were not diagnosed with IDA were included as the control group. The sample size determination comparing the means of PON-1 between two groups with effect size =0.6; α=0.05 and β=0,1, yielded a minimum sample size for each group of 45.[16]

The most important clinical indicators in the diagnosis of IDA for the inclusion of patients in the study were: age 40 years and over, hemoglobin concentration Hb <12 g/dl in women, transferrin saturation ≤15%, mean red blood cell volume <80 fL, serum iron concentration <45 g/dL, and serum ferritin concentration <15 ng/mL as an internationally valid criterion.[19]

The healthy females, who did not have the exclusion criteria and anemia, were included in the study as the control group. Exclusion criteria were: i) usage of any medication, ii) chronic disease, iii) oral or parenteral iron treatment in the last 3 months, iv) presence of dimorphic anemia, v) history of blood transfusion within 3 months before the study, vi) systemic or local infection, and liver and renal diseases.

Blood sample collection

The blood samples of the individuals included in the study were taken after 8 hours of fasting. The biochemistry panel was studied the same day, the blood sample taken for other markers was centrifuged at 5000 rpm for 5 minutes and the serum was separated and transferred to Eppendorf tubes. The separated sera were stored at -20^{o}C until the studies were carried out to determine the parameters that are indicators of oxidative stress.

Laboratory analysis

Complete blood counts of the individuals in the study group were studied with the Beckman Coulter LH 780 (Miami, Florida, USA) device. Iron, iron-binding capacity, and ferritin from the collected samples were determined by the enzymatic colorimetric method in an autoanalyzer (Olympus AU 2700, Mishima Olympus Co. LTD. Japan). Transferrin saturation was calculated using the formula: Serum iron / Serum iron-binding capacity x 100. Folic acid and vitamin B12 levels were measured in all patients to exclude vitamin B12 and folic acid deficiency.

Determination of oxidative stress parameters

Oxidant and antioxidant markers (TAS. TOS, OSI, PON-1, and MPO levels) were measured in the individuals constituting the study groups.

$$OSI = \frac{\text{TOS, } \mu\text{mol } H_2O_2 \text{ equiv./lt}}{\text{TAS, mmol Trolox equiv./lt X 10}}$$

The total antioxidant status (TAS)

The TAS of all individuals in the study group was determined spectrophotometrically using the automatic measurement method. With this test, the antioxidants in the samples reduce the dark blue-green, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS(*+)), ABTS radical to the colorless reduced ABTS form. The change in absorbance at 660 nm observed in this reaction is associated with the total antioxidant level of the sample. The results are expressed as millimolar hydrogen peroxide equivalents per liter (mmol Trolox equiv./L).

The total oxidant status (TOS)

The TOS in serum samples of individuals belonging to the study groups was determined spectrophotometrically using an automatic measurement method.[10] With this test, the oxidants in the sample oxidize the ferrous ion-o-dianisidine complex to the ferric ion, and the oxidation reaction here is prolonged by the glycerol molecules in the reaction medium. The ferric ion forms a colored complex with the chromogen xylenol orange in an acidic environment. Here, a measurable color intensity occurs and the total amount of oxidant molecules in the sample is determined with the help of a spectrophotometer. The results are expressed as micromolar hydrogen peroxide equivalents per liter (μmol H_2O_2 equiv./L).

The oxidative stress index (OSI)

The OSI, which is the indicator of oxidative load, was calculated according to the formula below and the results are shown in arbitrary units (AU).[19]

The paraoxonase-1

The PON-1 activity was measured using a kit, which consists of two different sequential reagents, namely Tris-buffer as a cofactor of PON-1 enzyme and a stable substrate solution. The sample is mixed with Reagent 1 and the substrate solution is added. The linear increase in absorbance of p-nitrophenol produced from paraoxon is examined. The non-enzymatic hydrolysis of paraoxon is subtracted from the total hydrolysis rate. Based on the molar absorptivity rate of p-nitrophenol of 18,290 M^{-1} cm^{-1}, one unit of PON-1 activity is equal to 1 mole of paraoxon hydrolyzed per liter per minute at 37°C.[20] The PON-1 results were expressed as U/L in serum.

The myeloperoxidase (MPO)

The spectrophotometric method was used to determine MPO activity. Myeloperoxidase activity is evaluated by measuring the H_2O_2-dependent oxidation of o-dianisidine. With this method, one unit of enzymatic activity is defined as the amount of MPO that causes a 1.0/min change in absorbance at 410 nm at 37°C.[19]

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 20.0 software (IBM SPSS Inc., USA). The normality of the resulting data distribution was investigated by the Shapiro-Wilk test. Since the significance level of each parameter with the Shapiro-Wilk test was $p<0.05$, it was decided that the distribution was not normal ($p=0.000$-0.016). For this reason, non-parametric Mann Whitney-U tests were used. The critical significance level was determined at 0.05. All data are reported as median and interquartile range (IQR).

Ethical clearance

For this study, we obtained written consent from the patients and approval from the local ethics committee of our hospital under no. 28.08.2019-32.

RESULTS

All demographic data belonging to patient and control groups are shown in Table 1. According to the findings, age parameters were not found to be significantly different between IDA patients and healthy individuals ($U=262.500$, $p=0.211$, $p>0.05$). However, for other all parameters, statistically significant differences between the patient group and control group were found ($p<0.05$). In our study, serum iron, transferrin saturation, and ferritin data were found to be significantly lower in patients with IDA compared to healthy individuals in the control group. In addition, there was a significant increase in iron-binding capacity among the related groups. When we compared the complete blood count (CBC) parameters, MCV, MCH, and MCHC were found to be significantly lower in the patients with IDA compared to the healthy individuals (Table 1). Oxidative stress parameter data of patients and controls are given

Table 1. Comparison of demographic and clinical parameters between patients with IDA and control group

Parameters	IDA (n:47) (Median, IQR)	Control (n:47) (Median, IQR)	p-value
Age (year)	37.0 (28.0-44.0)	31.0 (24.0-41.0)	0.211*
Hemoglobin (g/dL)	9.6 (7.2-11.0)	12.8 (12.5-13.8)	0.000
MCV (fL)	67.4 (60.4-77.5)	81.4 (78.783.3)	0.000
MCH (pg)	20.9 (16.8-23.9)	26.9 (24.5-28.0)	0000
MCHC (g/L)	30.1 (27.8-32.3)	33.2 (31.3-34.1)	0.000
Serum iron (ug/L)	18.0 (16.0-25.0)	57.0 (41.0-93.0)	0.000
TIBC (ug/dL)	446.0 (382.0-477.0)	370.0 (324.0-431.0)	0.001
Transferrin saturation (%)	4.0 (3.0-6.0)	23.0 (13.0-34.0)	0.000
Ferritin (ng/mL)	4.6 (2.2-8.7)	18.7 (9.9-42.2)	0.000

IDA: Iron deficiency anemia; IQR: Inter quartile range. Critical statistical significance level at p>0.05. MCV : mean corpuscular volume ;MCH : mean corpuscular hemoglobin ;MCHC : mean corpuscular hemoglobin concentration; TIBC : total iron-binding capacity

in Table 2 together with the statistical analysis data. A significant between-group difference was observed for TAS, TOS, and MPO using the Mann-Whitney U test (p<0.005). The TAS (1.67 mmol Trolox equiv./L) and MPO (89.0 U/L) levels in the control group were significantly higher than those in the IDA patient group [1.42 mmol Trolox equiv./L (p=0.000) and 54.00 U/L (p=0.019), respectively] (Table 2). However, the TOS level (6.44 μmol H_2O_2 equiv./L) and the OSI level (0.42 Arbitrary unit) of the patient group was significantly higher than that of the control group (4.13 μmol H_2O_2 equiv./L) and (0.24 Arbitrary unit , respectively (p=0.000). Moreover, for PON-1 data, a statistically non-significant difference was observed among these groups (p=0.375, which is greater than 0.05) (Table 2).

DISCUSSION

Iron deficiency anemia, one of the most common causes of anemia, is an important public health problem, especially in developing countries where nutritional problems are much more common.[20,21] This disease is mostly seen in women. Menstruation, pregnancy, abortion, and curettage are the most frequent causes in women. In menopausal women, the reason for the emergence of iron deficiency anemia is assumed to be located in the digestive system until proven otherwise.[22] In this study, we planned to compare some oxidative stress markers (TAS, TOS, serum MPO, and PON-1 levels) between patients with iron deficiency anemia and healthy controls. In our study, TAS levels were found to be statistically lower in the patient group.

Table 2. Median values of oxidative and antioxidants stress markers in the iron-deficiency anemia (IDA) and control group

Variables	IDA (n=47) (Median, IQR)	Control (n=47) (Median, IQR)	p-value
TAS (mmol Trolox Equiv./L)	1.42 (1.39-1.54)	1.67 (1.55-1.81)	0.000
TOS (μmol H_2O_2 Equiv./L)	6.25 (5.30-8.16)	4.13 (3.60-5.75)	0.000
OSI (Arbitrary unit)	0.42 (0.38-0.55)	0.24 (0.20-0.37)	0.000
MPO (U/L)	54.00 (38.0-74.0)	89.0 (53.0-120.0)	0.019
PON-1 (U/L)	272.00 (97.0-382.0)	147.0 (113.0-298.0)	0.375*

IQR: Inter Quartile range; TAS, total antioxidant capacity; TOS, total oxidant status; OSI, oxidative stress index; MPO, myeloperoxidase; PON-1, paraoxonase-1 activity; P=0.05, Critical statistical significance level at p>0.05

Some studies have included similar parameters. It is noteworthy that in most of the studies, oxidative stress increased, and antioxidant parameters decreased in these patients. In the case of oxidative stress, while SOD, glutathione peroxidase (GSH-Px), and glutathione reductase increase, antioxidant vitamins decrease, and TAS can determine the real effect.[23,24] In the case of IDA, the antioxidant capacities of erythrocytes are decreased, and lipid peroxidation is accelerated.[25] In IDA, increased oxidative stress, an inadequate antioxidant system (such as SOD, GSH-Px) and hypoxic conditions may be attributed to increased superoxide release because of mitochondrial function disorders in skeletal muscle, heart, liver, and blood cells.[26] In the study of Zaka-Ur-Rab et al.[27] levels of antioxidant enzymes were significantly lower in children with IDA as compared to controls.

In the study of Aslan et al.[18] oxidative stress was investigated in 26 patients with IDA and 20 healthy individuals. In this study, serum TAS was found to be significantly lower in IDA compared to healthy subjects. Myeloperoxidase is considered an important oxidant marker in many diseases. In the studies conducted, it is accepted that lipid peroxidation plays a role in the pathogenesis of various disorders and diseases.[28] Inhibition of lipid peroxidation, which causes changes in fluidity and permeability, inhibition of metabolic processes, and changes in ion transport and membrane organization, are important for antioxidants.[29] In our study, a significant between-group difference was observed for TAS, TOS, OSI, and MPO. Another antioxidant has been included in the studies recently, namely PON-1, which is a protective enzyme and an important molecule in the pathogenesis of many diseases.[30] In some studies, it has been observed that the level of PON-1, which is an important antioxidant marker of oxidant balance, is low in some diseases such as cardiovascular diseases, insulin resistance, and diabetes.[31,32] In a study by Okuturlar et al.[17] PON-1 activity was investigated in 50 patients with iron deficiency anemia (IDA) and 40 healthy individuals. They found that the mean PON-1 activity in the iron-deficiency anemia group was significantly lower than that of the control group, which was also found by Merono et al.[33] Similarly, in our study we observed a decreased level of PON-1 activity in patients with IDA. A statistically significant difference in PON-1 data was not observed among these groups. In another study, it was stated that the oxidant-antioxidant balance was disturbed, and oxidative stress occurred during the anemia process in individuals with iron deficiency. The investigators also stated that the inadequacy of the high antioxidant activity of erythrocytes in patients with iron deficiency in the process of anemia may be attributed to the release of superoxide, which creates mitochondrial dysfunctions in skeletal muscle, liver, heart, and blood cells.[34] An increase in oxidative stress may play a role in the pathophysiology of iron deficiency anemia. It has been stated that in individuals with iron deficiency anemia, antioxidant supplementation together with iron therapy may provide better responses and early improvement of the symptoms related to iron deficiency anemia.[18]

The present study has broad implications. Low hemoglobin and hematocrit associated with iron deficiency anemia are closely associated with poor quality of life and performance. This is important in terms of demonstrating the effectiveness of oxidative stress, which plays a role in the processes affecting activities of daily living, and of the role of antioxidant support in overcoming this stress. In addition, anemia also influences the cardiovascular system. Therefore, although these patients have risk factors for cardiovascular diseases, some biomarkers are important to obtain information about the process. On the other hand, low ferritin levels or transferrin saturation indicate an absolute or functional iron deficiency state. The difficulty of distinguishing chronic diseases accompanying iron deficiency anemia from the anemia itself limits this study. In future studies adding different

markers specific to iron deficiency anemia and oxidative stress is important in terms of the pathophysiology of this disease and the prevention of the development of other diseases.

CONCLUSION

This study demonstrated that adult women with IDA are subject to chronic oxidative stress. Therefore, it is important to determine the oxidative stress status from important markers to minimize the risk of IDA in adult women.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for this work.

AUTHOR CONTRIBUTIONS

EA contributed to writing the manuscript, EA and GAA contributed to design and data collection. AK and GAA contributed to analyzing the data. EA contributed to revising the manuscript. All authors have read and approved the final manuscript.

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