BODY IRON STORES AND OXIDATIVE STRESS MARKERS IN WOMEN OF REPRODUCTIVE AGE: IS IT RELATED TO ATHEROSCLEROSIS?

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Abstract

BACKGROUND: Elevated body iron stores have been suggested to be a risk factor for cardiovascular disease (CVD). We examined whether elevated plasma ferritin concentrations as indicator of iron stores, affect the oxidative stress markers in a reproductive age women population.

METHOD: One hundred sixty, 20-45-year-old women were randomly selected. We investigated body iron stores by measuring the concentrations of plasma ferritin. Furthermore, we assessed oxidative stress markers by measuring the concentrations of plasma malondialdehyde (MDA) and activities of erythrocyte cytoprotective enzymes, including superoxide dismutase (CuZn-SOD), catalase (CAT) and glutathione peroxidase (GPX) in a random sample of cardiovascular disease-free women in reproductive age.

RESULTS: Subjects in the highest tertile of plasma ferritin presented the highest levels of plasma MDA ($p<0.001$) and CAT activity ($P < 0.05$). Furthermore, these Subjects presented the lowest levels of CuZn-SOD activity ($P < 0.01$). No significant associations were found between the tertile of plasma ferritin in GPX activity. Plasma ferritin was significantly directly associated with plasma MDA levels and inversely associated with CuZn-SOD activity. Using multiple regression, Plasma ferritin levels was positively correlated with MDA levels and inversely correlated with CuZn-SOD activity.

CONCLUSION: Our findings revealed an association between body iron stores and oxidative stress markers linked to atherosclerosis process. The results emphasize that iron overload would elevate the risk of coronary artery disease by promoting the lipid peroxidation.

Keywords: Iron stores, ferritin, oxidative stress, atherosclerosis, women, reproductive age.

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Introduction

Interest in iron overload has increased both in the scientific and public health communities. Meanwhile, the proportion of iron-replete individuals in industrialized countries has also risen.[1] The free iron noxious to cells because it catalyzes the generation of hydroxyl radicals (•OH) from superoxide (O_2•-) and hydrogen peroxide (H_2O_2) via the Fenton reaction.[2] The potential toxicity of iron derives from its ability to serve as a catalyst in oxidation-reduction reactions, and its toxicity is enhanced by the limited capacity of the human body to excrete iron.[3]

There are several risk factors which their association with atherosclerosis is well established, including age, gender, lipid disorders, smoking, hypertension, diabetes mellitus, obesity, and sedentary lifestyle.[4,5] Yet, about 25% of cardiovascular diseases (CVDs-) related deaths in men and 15% in women occur in persons with multivariate Framingham Study risk factors.[6] It has been suggested that about half of atherosclerosis cases cannot be

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attributed to standard risk factors.[4] This fact has recently stimulated a call for investigation of new risk factors.[7,8] In the last decade evidence showed that a crucial and causative role in the pathogenesis of atherosclerosis, is played by the free radical process known as lipid peroxidation. It is currently believed that lipid peroxidation is involved in the oxidative modification of low density lipoprotein and this ultimately results in the formation of atherosclerotic lesions.[9,10]

It has been suggested that the risk of coronary heart diseases increases with increasing body iron stores. In support of that hypothesis, a prospective epidemiological study of heart disease in Finnish men found that the risk of heart attack, increased with increasing levels of serum ferritin.[11] However, other studies have failed to support the hypothesis that high body iron stores increase the risk of coronary heart disease.[12,13] Serum ferritin levels reflect the iron stores in the body. Free radical formation and lipid peroxidation can be prevented by the iron-chelating agent desferrioxamine.[14] Cellular defense mechanisms against superoxides include series of linked enzyme reactions which remove the toxic radicals and repair radical-induced damage. The first of these enzymes is copper zinc superoxide dismutase (CuZn-SOD) which converts the superoxide anion into hydrogen peroxide. Hydrogen peroxide, also toxic to cells, is removed by catalase (CAT).[15] A convenient and sensitive method for estimation of lipid peroxide concentration is the quantitative estimation of their metabolic end-product malondialdehyde (MDA) using the thiobarbituric acid method. MDA is one of the most frequently used indicators of lipid peroxidation.[16]

The purpose of this study is to investigate whether body iron stores have any relationship with levels of oxidative stress markers. To test this hypothesis, we investigated body iron stores by measuring the concentrations of plasma ferritin. Furthermore, we assessed oxidative stress markers by measuring the concentrations of plasma malondialdehyde (MDA) and activities of erythrocyte cytoprotective enzymes (including, CuZn-SOD, CAT and glutathione peroxidase) in a random sample of cardiovascular disease-free women in reproductive age.

Materials and Methods

The subjects were selected from women receiving rural health centers services in Kerman Province, Iran. 160 women, aged 20-45 years (mean age: 31.5 years) were randomly selected. To minimize the potential for confounding of the primary relation of interest (plasma ferritin and oxidative stress markers), We excluded pregnant and lactating women, those with history of cancer, cardiovascular disease, diabetes, renal or liver diseases, and those were taking vitamin or mineral supplements. Informed written consent was obtained from subjects before the study. Demographic data were gathered from samples using questionnaires and face-to-face interviews.

Body weight and height were measured for each subject and BMI was calculated. To calculate waist-to-hip ratio (WHR), the waist and hip circumferences were also measured.[17]

Venus blood samples were collected from the median cubital vein into standard tubes containing ethylene diamine tetra acetic acid (EDTA). Blood samples were centrifuged at 3000 rpm for 10 minutes at 4ºC and plasma was separated for MDA assay. The buffy coat was removed and the remaining erythrocytes were washed three times in cold saline (9.0 g/l NaCl) and hemolyzed by the addition of cold deionized water. The subjects' plasma and hemolysate samples were stored at -70ºC until analysis.

Plasma MDA concentrations were assayed by measurement of thiobarbituric acid reactive substances (TBARS) according to Satoh method.[17] The pink chromogen produced by the reaction of thiobarbituric acid with MDA was measured at 530 nm. In order to express the activities enzymes per gram hemoglobin (Hb), Hb concentration was measured in the hemolysates with a standard kit involving the cyanmethhemoglobin method (Drabkin's method).

Catalase (CAT, E.C.1.11.1.6) activity was determined according to Hygo Aebi.[18] Activity of CAT was determined by following the decomposition of H2O2 in phosphate buffer pH 7.2 spectrophotometrically at 230 nm.

Glutathione peroxidase (GPX, E.C.1.11.1.9) activity was measured according Paglia and Valentine method 19 and superoxide dismutase (SOD, E.C.1.15.1.1) activity was assayed by kit RAN-SOD (cat.NO.SD 125). Plasma ferritin concentrations were determined with Radioimmunoassay method and standard kit with the Ciba Corning ACS-180 analyzer.

In present study, the 25th and 75th percentile values of plasma ferritin were used as threshold values to define 3 categories of iron stores: low (< 25th percentile), medium (25th to 75th percentile), and high (> 75th percentile). The oxidative stress markers of interest were analyzed as continuous variables. Goodness of fit to normal distribution was investigated by probit plots and the Kolmogrov test. Comparison between mean values of oxidative stress markers across groups of iron stores were performed by the calculation of one-way analysis of variance (ANOVA). Pearson's correlation coefficient was applied to assess relationships between plasma ferritin and oxidative stress markers. Because the oxidative stress markers of interest, represent a cluster of indicators that collectively predict risk and may be correlated, we used multivariate analysis of variance in which the entire set of oxidative stress markers was considered as the dependent variable. A single P value was used to interpret the significance of the association between the set of oxidative stress markers and plasma ferritin concentration. This approach may be superior to consideration of each oxidative stress marker separately, because it adjusts for multiple comparisons and accounts for co-linearity between the dependent variables. Because 2 comparisons were done (high versus medium and low versus medium plasma ferritin), a Bonferroni correction was applied. P values less than 0.05 were considered as statistically significant. SPSS software (Statistical Package for Social Sciences, Version 12.5, SPSS Inc, Chicago, IL. USA) used for all statistical calculations.

Results

The 25th and 75th percentile values for plasma ferritin (which were used to define low, medium, and high iron stores) were 23 and 68 μg/L for participants.

Mean values of oxidative stress markers according to tertiles of plasma ferritin concentration are shown in table 1. Subjects in the highest tertile of plasma ferritin presented the highest levels of plasma MDA ($P < 0.001$). Those in the highest tertile of plasma ferritin had also the highest mean values of CAT activity ($P < 0.05$). Furthermore, Subjects in the highest tertile of plasma ferritin presented the lowest levels of CuZn-SOD activity ($P < 0.01$). Those in the highest tertile of plasma ferritin had also the lowest GPX activity but differences were not statistically significant.

Figure 1 shows the relationship between plasma ferritin with MDA levels (Figure 1A) and CuZn-SOD activity (Figure B). Plasma ferritin has significant direct association with plasma MDA levels ($r = 0.39$, $P < 0.0001$) and inverse association with CuZn-SOD activity ($r = –0.25$, $P < 0.001$). Figure 1 illustrates these relationships. Adjusted mean values for the selected oxidative stress markers in women in the lowest and highest tertiles of plasma ferritin compared with those in the middle tertile are shown in table 2 for the 2 models. In the first model, we controlled for age only. In the second model, we also controlled for BMI, WHR and number of pregnancies. We observed that the relationships remained significant even after adjustment for confounding. Plasma MDA levels was significantly higher in the highest tertile of plasma ferritin compared with the middle tertile ($P < 0.01$ with the Bonferroni adjustment for multiple comparisons). Furthermore, CuZn-SOD activity was significantly lower in the highest tertile of plasma ferritin compared with the middle tertile ($P < 0.01$ with the Bonferroni adjustment for multiple comparisons). No associations were found between the tertiles of plasma ferritin in GPX activity.

Discussion

In this study we measured plasma levels of ferritin which is in equilibrium in body stores and reflects the variations in the quantity of iron in the storage compartment, and MDA, a stable product of lipid peroxidation as an indicator of free radical generation in the human body. We investigated the relationship between ferritin and MDA.

The generation of free radicals is dependent on the presence of various transition metal ions.14 The most important transition metals in vivo are believed to be iron and copper. To support the possible role of iron in the generation of free radicals in vivo, it has been shown that coronary reperfusion damage, a process thought to be partially mediated by reactive oxygen species, has been shown to be increased by iron load, and this damage was partially reversed by iron-chelating agents in experimental animal models.[20] The mostprobable explanation for the effect of iron is, oxidation stimulating of low density lipoproteins (LDL).[14]

Table 1. Mean values of oxidative stress markers according to tertiles of plasma ferritin concentration

Oxidative stress markers	Plasma ferritin tertiles (µg/l)		
	Low (< 23) (n = 62)	Medium (23-68) (n = 56)	High (> 68) (n = 42)
MDA (µmol/l)	1.13 ± 0.14	2.39 ± 0.92*	3.47 ± 1.02*
CuZn-SOD (U/gHb)	836 ± 68	673 ± 56	587 ± 71 #
GPX (U/gHb)	98.1 ± 3.3	93.2 ± 4.1	89.7 ± 3.6
CAT (K/gHb)	165 ± 54	198 ± 47	219 ± 58†

Values are mean ± SD
* P < 0.001 compared with the lowest tetiles of plasma ferritin.
# P < 0.01 compared with the lowest tetiles of plasma ferritin.
† P < 0.05 compared with the lowest tetiles of plasma ferritin.

Table 2. Comparison of adjusted estimates (± SE) of selected oxidative stress markers by tertiles of plasma ferritin among women of reproductive age (20-45 y)

Plasma ferritin tertiles (µg/l)	MDA (µmol/l)	CuZn-SOD (U/gHb)	GPX (U/gHb)	CAT (K/gHb)
Model 1*				
Low (< 23)#	–2.264 ± 0.52	0.632 ± 0.028	0.021 ± 0.06	–1.652 ± 0.61
Medium (23-68)†	____	____	____	____
High (> 68)#	1.05 ± 0.37	–0.841 ± 0.044	–0.038 ± 0.02	0.94 ± 0.07
Model 2**				
Low (< 23)#	–1.167 ± 0.032	0.325 ± 0.025	0.041 ± 0.03	–0.984 ± 0.021
Medium (23-68)†	_____	_____	_____	_____
High (> 68)#	1.149 ± 0.481	–0.576 ± 0.001	0.022 ± 0.01	1.357 ± 0.742

† Reference category
* Adjusted for age
** Adjusted for age, body mass index (BMI) and waist-to-hip ratio (WHR) and number of pregnancies
# P < 0.01 (Bonferroni adjusted value for multiple comparisons) compared with medium plasma ferritin by multivariate analysis of variance in which the entire set of oxidative stress markers was considered as dependent variable.

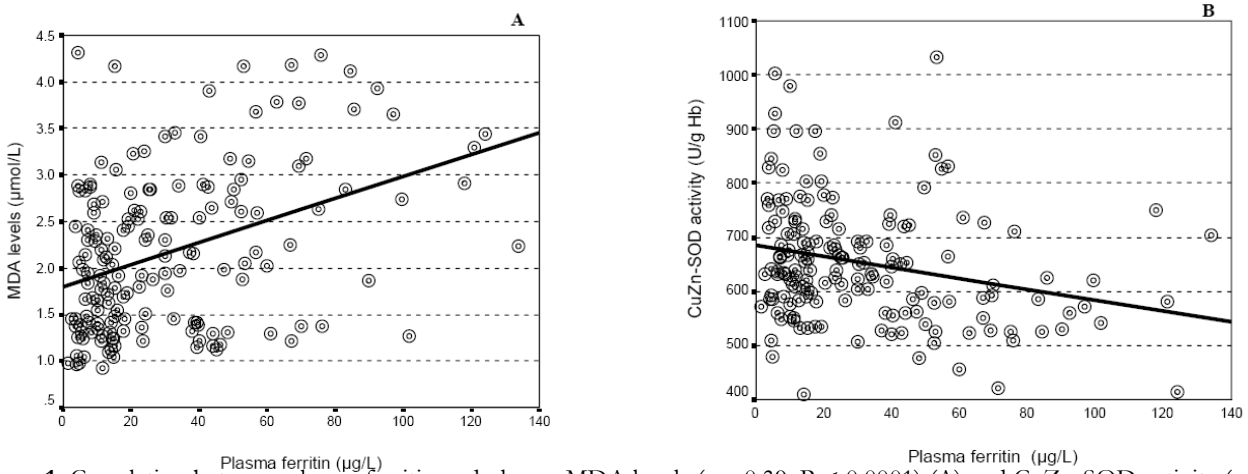

Figure 1. Correlation between plasma ferritin and plasma MDA levels (r = 0.39, P < 0.0001) (A) and CuZn-SOD activity (r = –0.25, P < 0.001) (B)

During the last few years, oxidative modification of LDL has come into focus as an important step in pathogenecity of LDL, in atherosclerotic lesions.[10] There is considerable experimental evidence indicating that this modification increases the atherogenic effects of the LDL molecule. Several epidemiological studies have shown dietary antioxidants (especially vitamin E) are inversely correlated with the incidence of coronary artery disease.[21,22]

Studies investigating whether iron status can be considered as a cardiovascular risk factor, which causes conflicting results, as reviewed recently.[23,24] This was not unexpected because none of the indicators of iron status evaluated such as hemoglobin, hematocrit, serum iron, transferrin, transferrin saturation, total iron binding capacity accurately reflects body iron.[25] Because plasma ferritin concentrations are directly proportional to intracellular ferritin concentrations, it is considered to be the best clinical measure of body iron stores25 and the most feasible to use in epidemiologic studies.[26] However, so far only a few studies have evaluated plasma ferritin concentrations to examine whether body iron stores are associated with cardiovascular diseases. Plasma ferritin concentrations are known to increase in response to inflammation. To decrease the confounding effect of inflammation on Plasma ferritin concentrations, we excluded subjects who had inflammation or other diseases.

A recently published study on the relation between serum ferritin and the risk of myocardial infarction suggests that iron might be important as a prooxidant in atherogenesis.[11] In our study, elevated Plasma ferritin concentrations were also associated with increase of oxidative stress. Similarly, Yesilbursa et al found positive relation between Plasma ferritin concentrations and plasma MDA concentrations in patients with coronary artery disease.[27]

Extensive reviews have been recently published, concerning the role of iron in free radical reactions, such as lipid peroxidation.[14,28] In one of the previous reports of our study on reproductive age women; we have also shown that subjects in the highest tertile of plasma iron presented the highest levels of plasma MDA.[29] Iron in body largely stored in ferritin. In essence, ferritin acts as a critical antioxidant defense by sequestering unbound or "free" iron, thereby limiting its participation in damaging oxidative reactions. Iron loading at the cell level induces a 10-fold increase in intracellular ferritin.[30] The induction of ferritin in endothelial cells is cytoprotective against oxidative injury by sequestering and inactiving iron. To promote free radical production, iron must be liberated from ferritin, but body iron is so tightly bound that there may not be free iron available in vivo under physiological conditions. It is believed that oxidant stress itself can provide the iron necessary for formation of reactive oxygen species, for example, by mobilizing iron from ferritin.[27] For instance, Superoxide radicals (O_2–) have been observed to liberate iron from ferritin by reducing ferritin-bound Fe+3 to Fe+2, whereupon it is released from ferritin and becomes available to catalyze self-propagating burst of oxidation.[31] The O_2– derived from stimulated granulocytes or generated by xanthine oxidase is able, in this way, to mobilize iron from ferritin.[32] Iron can also be released from ferritin at low extracellular PH or by arterial wall damage.[33,34]

We then investigated the relationship between body iron stores with the activities of erythrocyte antioxidant enzymes. We observed that subjects in the highest tertile of plasma ferritin levels had the lowest level of CuZn-SOD activity and highest level of CAT activity. To our knowledge, this is the first study to investigate the relation between plasma ferritin levels with the activities of erythrocyte antioxidant enzymes in an apparently healthy human population, and therefore, we could not compare our data with other epidemiological studies. Nevertheless, we did not observe changes of plasma iron with the GPX activity. However, some researchers observed increased CuZn-SOD activity in patients with iron deficiency anemia who had lower iron stores.[35,36] It is well known that reactive oxygen species, especially hydrogen peroxide, inhibit SOD activity.[36] Furthermore, decreased SOD activity may contribute to free radical production. CAT is an iron-dependent enzyme and would be expected to be increased with elevation of body iron stores.

Although the cross-sectional design of this study precludes inferences of causality, it is not likely the observed associations confounded by all factors which were controlled in the analysis. The choice of plasma ferritin as a valid indicator of iron stores is a potential concern. Finally, other strength of the study design was that the study sample consisted of relatively healthy women of reproductive age.

Our study may have some limitations in data gathering like all cross-sectional studies. First, like

all observational studies, our results could be biased by unrecognized confounders. Second, we couldn't assess nutrient intakes (including antioxidant nutrients) of participants.

In conclusion, we observed plasma ferritin concentrations, is associated with increased oxidative stress. The results are also in agreement with this concept that iron overload would elevate the risk of coronary artery disease by promoting the lipid peroxidation. Prospective studies, especially interventional studies that prepare adequate iron status, are needed to compare two possible scenarios: improved iron status increases the risk of CVD, or women with a higher risk of CVD have higher iron stores, with both factors resulting from the same underlying cause.

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