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Original Article

Oral iron supplementation leads to oxidative imbalance in anemic women: A prospective study

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SUMMARY

Background & aims: This study was aimed to assess the desirable and undesirable effects of iron (100 mg/day as ferrous sulphate) and folic acid (500 μg/day) supplementation in iron deficient anemic women.
Methods: Iron and folic acid supplementations were given to 117 anemic women (mild = 55, moderate = 40, and severe = 22) and 60 age matched placebo treated (100 mg cane sugar) non-anemic controls for 100 days. Blood index values, oxidative stress parameters, antioxidant enzymes and vitamins were estimated as per standard protocols.
Results: Haemoglobin (Hb) levels along with antioxidant enzymes, namely catalase, superoxide dismutase (SOD), glutathione reductase (GSH-Rd), reduced glutathione (GSH) and total antioxidant capacity (TAC) were found significantly increased ($P < 0.01$) in anemic women after treatment. However, the glutathione peroxidase (GSH-Px) and antioxidant vitamins A, C and E were found significantly decreased ($P < 0.01$) in all treated groups. Lipid peroxide levels (LPO), protein carbonyl (PC), conjugated dienes (CD), lipid hydroperoxide (LOOH) and oxidized glutathione (GSSG) levels were found significantly increased ($P < 0.01$) after oral iron supplementation groups. Moreover, undesirable side effects of iron supplementation were observed maximally in mild as compared with moderate and severe anemic groups, whereas nausea, vomiting, systemic reactions were negligible in all treated subjects.
Conclusion: Study found recommended dose of iron effective for improving Hb, but at the cost of increased oxidative stress (mild > moderate > severe). It is suggested that blind iron supplementation should be avoided and shall be provided on need basis.

1. Introduction

Iron deficiency anemia (IDA) is one of the most common nutritional disorders worldwide, affecting people of all ages both in developed and developing countries.[1] World Health Organization (WHO) reports indicate that the numbers are quite staggering: two billion people i.e. over 30% of the world's population are anemic with about one billion suffering from iron deficiency anemia. Anemia is one of the 15 leading contributors to the global burden of disease.[2] One study reveals that about 50–60% of pregnant females and 20–35% of non-pregnant females in developing countries are affected by iron deficiency anemia.[3] There are also reports that iron deficiency can lead not only to anemia but it may also impair work performance, lead to an abnormal neurotransmitter function and result in altered immunological and inflammatory defences.[4] Moreover, most non-pregnant women of reproductive age have lower than desirable iron stores or none at all making them more vulnerable to iron deficiency when iron intake is lowered or need increases.

Oral iron supplementation is a commonly used strategy to meet the increased requirements of risk groups, such as women of childbearing age. However, if provided in excess may induce peroxidative damage through production of reactive oxygen species

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(ROS) including loss of functional integrity, and decreased turnover of epithelial cells, as also with marked mucosal cell death.[5,6] Moreover, iron mediated oxidative damage has been demonstrated in vivo in normal red blood cells.[7]

On going through the available literature there seems to limited and contradictory data available on oxidative stress and antioxidant defense parameters in iron deficient anemic women and also on the effect of iron supplementation on antioxidant status. In contrast, Walter et al[8] showed increased mitochondrial peroxidative damage in iron deficiency and iron overloaded rats. The present study was designed to evaluate the markers of oxidative stress and the activities of antioxidative enzymes in iron deficient anemic women and their response to oral iron supplementation.

2. Materials and methods

2.1. Subjects

The present study comprised a total of 177 non-pregnant women, aged 20–40 yrs. Out of 177, 60 were non-anemic (Hb > 11 g/dl) women (controls) and 117 were anemic (mild = 55, moderate = 40 and severe = 22) women (cases). The subjects were selected amongst those attending the out patient Department of Obstetrics and Gynaecology, Queen Mary's Hospital, Chhattrapati Shahuji Maharaj Medical University, Lucknow, U.P., India. The controls taken for the study were not related to the cases. Care was taken to ensure that all the subjects belonged to middle socioeconomic group (who were able to meet the basic necessities of life and have same requirements of comfort). Selected subjects were all consumers of normal mixed Indian diet, not taking any drugs for preceding one month. The inclusion criteria of anemic subjects were according to WHO, which defines mild anemia as Hb 10.0–10.9 g/dl, moderate as Hb 7.0–9.9 g/dl and severe as Hb < 7.0 g/dl.[9] Exclusion criteria were Hb less than 6.5 g/dl, no Hb rise by 1% after 3-week of iron–folic supplementation, alcoholics, smokers, and those suffering from metabolic diseases like diabetes mellitus, malignancy, heart disease, infections such as tuberculosis, HIV, and those who were regularly using minerals/vitamins supplements or suffering from endocrine disorders.

Informed consent was obtained from each subject and the study was approved by the Institutional Ethical Committee of Chhattrapati Shahuji Maharaj Medical University, Lucknow, India.

2.2. Treatment

At recruitment, all women (non-anemic and anemic) were first dewormed by giving them a single dose of Albendazole following Metronidazole (400 mg) three times daily for five days. Two days later i.e. after one week of recruitment, anemic women were given iron supplements (100 mg as ferrous sulphate and 500 μg folic acid) orally once a day, daily for 100 days; similarly controls were given placebo capsules containing 100 mg cane sugar orally once a day, daily for 100 days.

Before deworming, venous blood of all women (non-anemic and anemic) was taken for the estimation of biomarkers of iron status, antioxidant status and oxidative stress parameters (pre-treatment). All tests were repeated after 100 days of treatment (post-treatment).

After three weeks of iron supplementation, Hb was assessed. Subjects, who's Hb was not improved, were excluded from the study and were referred for other investigations. All blood collections were done in the Department of Obstetrics & Gynaecology, Queen Mary's Hospital, Chhattrapati Shahuji Maharaj Medical University, Lucknow. The subjects were instructed not to change their dietary or daily activities during the study.

2.3. Sample collection

6 ml venous blood was taken from each subject and divided into three aliquots at the recruitment. 2 ml blood was transferred to an EDTA containing evacuated tube used to determine Hb, MCH, MCV, RBCs count, GSH and GSSH. 2 ml of whole blood was also transferred into EDTA containing tube and then centrifuged; plasma separated and used for the estimation of LPO, PC, CD LOOH and iron. Remaining 2 ml of venous blood was also centrifuged at 3000 rpm for 15 min, serum separated and used for the estimation of vitamin A, C and E. The RBCs was lysed by mixing chilled water and RBC lysate was used for the estimation of antioxidant enzymes namely catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd).

2.4. Analytical estimation

2.4.1. Measurement of blood index parameters

Blood haemoglobin was determined by using the cyanomethemoglobin method.[10] Haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), red blood cell counts were determined by using Sysmax A-380 automated cell counter. The concentration of iron in plasma was measured with flame atomic absorption spectrophotometer (Perkin Elmar AAS-700 Ueberlinger, Germany).[11]

2.4.2. Measurement of endogenous antioxidants

Catalase (CAT, EC 1.11.1.6) activity was assayed as per the method of Aebi et al[12] using hydrogen peroxide as substrate; the decomposition of H_2O_2 was followed at 240 nm on spectrophotometer. The CAT activity was expressed as nmole H_2O_2 catabolized/min/mg protein. The superoxide dismutase (SOD EC 1: 15.1.1) activity was determined from its ability to inhibit the reduction of NBT in presence of PMS according to the method of McChord and Fridovich.[13] The reaction was monitored spectrophotometrically at 560 nm. The SOD activity was expressed as U/mg protein (1 unit is the amount of enzyme that inhibit the reduction of NBT by one half in above reaction mixture). The glutathione peroxidase (GSH-Px, EC 1.11.1.0) was assayed by the method of Pagila and Valentine[14] using GSH, NADPH and H_2O_2 as reactants. The activity of GSH-Px was expressed as nmoles of NADPH oxidized/min/mg protein. The GSH-Rd was assayed by the method of Hazelton & Lang.[15] The activity of GSH-Rd was expressed as nmoles of NADPH oxidized/min/mg protein. Total protein of RBC sample was determined by the method of Lowry et al.[16] GSH and GSSG were measured using 5,5′-dithiobis-2 nitrobenzoic acid (DTNB) as described by Ellman et al.[17]

Ascorbic acid (vitamin C) levels were estimated as described by Beulter.[18] Retinol (vitamin A) and α-Tocopherol (vitamin E) were measured by high-performance liquid chromatography (HPLC) as per the modified method of Omu et al.[19] Briefly α-tocopherol acetate and retinol acetate were pipetted into an eppendrof tube. Into this, blood serum was added and vortex mixed; hexane extract of vitamin A and E was aspirated out in a glass tube, dried under nitrogen stream, and dissolved into methanol. Finally, this preparation was injected into HPLC filled with a reverse phase C-18 stainless steel column. The vitamins were eluted with methanol at the flow rate of 1.5 mL/min for 15 min. The peak heights and the curve areas of vitamin A and E and their acetate were measured to calculate the amount of these vitamins in blood serum in an ultraviolet detector with 292 nm filters. Total antioxidant capacity (TAC) was estimated by ferric reducing ability of plasma (FRAP Assay) where antioxidant power converts ferric to ferrous ion reduction at low pH causing a coloured ferrous tripyridylfriazine complex.[20]

2.4.3. Measurement of lipid peroxidation and protein oxidation

The lipid peroxide (LPO) levels were measured by the method of Okhawa et al.[21] The LPO content in sample was measured as thiobarbiuteric acid reactive substance (TBARS). Acetic acid detaches the lipid and protein of the sample. The protein in the reaction mixture is dissolved by the addition of sodium dodecyl sulphate (SDS). Thiobarbiuteric acid (TBA) reacts with lipid peroxides, lipid hydroperoxides and unsaturated fatty acid and form the colour adduct were then estimated spectrophotometrically at 532 nm and expressed as nmole of MDA/mg protein. Lipid hydroperoxide (LOOH) levels were measured by the method of Haldebrandt and Roots.[22] In this method, ferrousthiocynate was measured on Fe^{2+} to Fe^{3+} by H_2O_2 and resulted in intense pale colour, which was read at 480 nm. Standard cumine hydroperoxide was used as a control and results were expressed as mmole cumine hydroperoxide per liter. Conjugated dienes (CD) were measured by the method of Racknagel and Ghosal.[23] The cytosolic fraction was extracted from long chain fatty acids by chloroform: methanol. The absorbance of lipid extract was read at 220 nm on spectrophotometer. The level was expressed as μmol using the molar extinction coefficient of 2.1×10^3. The protein oxidation was measured by estimating the protein carbonyl (PC) levels by the method of Liu et al.[24] Protein carbonyl content was determined in the samples by measuring the 2, 4-dinitrophenylhydrazine (DNPH) adducts at 375 nm. Carbonyl contents were calculated by using a molar extinction coefficient (e) of 22,000 M^{-1} cm^{-1}. Data were expressed as nmoles carbonyl/mg.

2.4.4. Statistical analysis

Groups were compared according to an intention-to-treat strategy. The pre-treatment levels of all four groups (control, mild, moderate and severe) were compared together by one-way analysis of variance (ANOVA) and the significance of mean difference of control group with other groups was done by Dunnett's post hoc test. The pre and post-treatment level of each group was compared by paired t-test. Differences in proportions were done by chi-square test. A two-tailed ($\alpha = 2$) probability (P) value $P < 0.05$ was considered to be significant. GraphPad Prism (version 3) and STATISTICA (version 6) were used for the analysis.

3. Results

3.1. Blood profile

The pre and post-treatment blood index parameters of all non-pregnant healthy and anemic women are summarized in Table 1. The pre and post-treatment mean values of blood parameters in control were found to be similar i.e. did not differed significantly ($P > 0.05$). However, the pre-treatment mean values of all blood parameters in all anemic groups were found to be significantly ($P < 0.01$) lower as compared to controls. Treatment of these anemic women with elemental iron and folic acid (100 mg and 500 μg/d) for 100 days showed a significant ($P < 0.01$) reversal of the above parameters. Treatment with oral iron significantly increased the blood Hb in mild (10.48 ± 0.03 vs. 12.18 ± 0.11; $P < 0.01$), moderate (8.43 ± 0.14 vs. 10.93 ± 0.12; $P < 0.01$) and severe anemic women (6.80 ± 0.04 vs. 9.89 ± 0.11; $P < 0.01$) compared with the pre-treated groups. Furthermore, hematocrit was also significantly increased in mild (32.14 ± 0.34 vs. 36.11 ± 0.37; $P < 0.01$), moderate (28.13 ± 0.41 vs. 33.16 ± 0.50; $P < 0.01$) and severe anemic women (23.22 ± 0.53 vs. 28.55 ± 0.48; $P < 0.01$) after 100 day of treatment with iron and folic acid. The mean corpuscular volume increased significantly ($P < 0.01$) in mild, moderate and severe anemic women as compared with respective pre-treatment levels. The plasma iron (Fe) level of controls was 47.50 ± 0.74 mg/dl. On the other hand, we found significantly decreased level of Fe in mild (20.2%; $P < 0.01$), moderate (43.4%; $P < 0.01$) and severe (58.4%; $P < 0.01$) anemic patients in all the pre-treated groups compared with controls. After treatment, the levels of Fe increased significantly in mild (23.8%; $P < 0.01$), moderate (24.6%; $P < 0.01$) and severe (28.1%; $P < 0.01$) anemic subjects when compared with pre-treated groups.

3.2. Antioxidant enzymes and vitamins

The pre and post-treatment antioxidant enzymes and vitamins of all non-pregnant healthy and anemic women are summarized in Table 2. Like, blood index parameters, the pre and post-treatment mean values of all antioxidant enzymes and vitamins in control were found to be similar ($P > 0.05$). Table 2 showed that the pre-treatment mean levels of catalase in the RBC of non-pregnant non-anemic women were 56.15 ± 108 nmole H_2O_2 catabolized/min/mg protein. The pre-treatment catalase was decreased significantly ($P < 0.10$) in the RBC of mild (44.98 ± 0.73 U/mg protein), moderate (39.60 ± 0.83 U/mg protein; $P < 0.01$) and severe anemic women (33.20 ± 0.91 U/mg protein; $P < 0.01$) as compared to pre-treatment mean level of catalase of control group. A significant reversal in the level of CAT was observed in mild (51.95 ± 0.71 U/mg protein; $P < 0.01$), moderate (45.38 ± 0.84 U/mg protein; $P < 0.01$) and severe (38.95 ± 1.01 U/mg protein; $P < 0.01$) anemic women after treatment with iron and folic acid. The pre-treatment mean levels of superoxide dismutase, glutathione reductase, reduced glutathione and oxidized glutathione of control were 1.19 ± 0.03 U/mg protein, 28.83 ± 0.78 nmole NADH oxidized/min/mg protein, 417.20 ± 2.81 μM and 144.82 ± 1.75 μM, respectively. The pre-treatment mean levels of these parameters especially in moderate and severe groups were found to be significantly ($P < 0.01$) lower as compared to respective controls. Treatment with iron and folic acid significantly ($P < 0.05$ or $P < 0.01$) recovered the levels of SOD in mild, moderate and severe anemic women compared with pre-treated levels. Similarly treatment also significantly ($P < 0.01$) recovered the levels of glutathione reductase, reduced glutathione and oxidized glutathione compared with pre-treatment levels. The level of

Table 1
Blood index values (Mean ± SE) of pre and post-treated healthy and anemic women.

Parameters	Control ($n = 60$)		Mild ($n = 55$)		Moderate ($n = 40$)		Severe ($n = 22$)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Hb (g/dl)	13.45 ± 0.16	13.56 ± 0.17	10.48 ± 0.03[a]	12.18 ± 0.11[ab]	8.43 ± 0.14[a]	10.93 ± 0.12[ab]	6.80 ± 0.04[a]	9.89 ± 0.11[ab]
Hct (%)	36.66 ± 0.43	36.92 ± 0.46	32.14 ± 0.36[a]	36.12 ± 0.40[b]	28.13 ± 0.41[a]	33.16 ± 0.50[ab]	23.22 ± 0.53[a]	28.55 ± 0.48[ab]
MCV (fl)	85.91 ± 0.99	86.42 ± 1.06	77.29 ± 1.25[a]	84.13 ± 1.19[b]	71.16 ± 1.35[a]	78.56 ± 1.20[ab]	67.39 ± 1.68[a]	72.25 ± 1.39[ab]
MCH (pg)	30.71 ± 0.36	30.97 ± 0.38	28.61 ± 0.50[a]	30.45 ± 0.61[b]	27.22 ± 0.66[a]	29.32 ± 0.35[b]	25.15 ± 0.64[a]	27.23 ± 0.51[ab]
RBC ($\times 10^{12}$/L)	4.86 ± 0.06	4.78 ± 0.07	4.28 ± 0.06[a]	4.79 ± 0.06[b]	3.74 ± 0.04[a]	4.31 ± 0.06[ab]	3.63 ± 0.05[a]	3.96 ± 0.06[ab]
Fe (mg/dl)	47.50 ± 0.65	47.23 ± 0.66	37.88 ± 1.06[a]	46.92 ± 0.94[b]	26.84 ± 1.07[a]	33.45 ± 0.98[ab]	19.73 ± 0.57[a]	25.28 ± 0.86[ab]

[a] $P < 0.01$ in comparison with pre control; [b] $P < 0.01$ comparison between pre and post.

Table 2
Blood antioxidant parameters (Mean ± SE) of pre and post-treated healthy and anemic women.

Parameters	Control ($n = 60$)		Mild ($n = 55$)		Moderate ($n = 40$)		Severe ($n = 22$)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
CAT	56.15 ± 1.08	56.34 ± 1.10	44.98 ± 0.73[a]	51.95 ± 0.71[a]	39.60 ± 0.83[a]	45.38 ± 0.84[ab]	33.20 ± 0.91[a]	8.95 ± 1.01[ab]
SOD	1.19 ± 0.03	1.17 ± 0.03	1.08 ± 0.02[a]	1.18 ± 0.02[b]	0.96 ± 0.02[a]	1.03 ± 0.04[ab]	0.75 ± 0.03[a]	0.86 ± 0.03[ab]
GSH-Px	37.89 ± 0.63	37.72 ± 0.63	35.86 ± 0.73	35.19 ± 0.69[ab]	28.60 ± 0.63[a]	26.73 ± 0.58[ab]	25.14 ± 1.20[a]	22.14 ± 0.90[ab]
GSH-Rd	28.83 ± 0.78	28.95 ± 0.77	26.13 ± 0.62[a]	28.53 ± 0.62[b]	25.25 ± 0.70[a]	27.13 ± 0.72[b]	21.34 ± 0.90[a]	22.95 ± 0.92[ab]
GSH	417.20 ± 2.76	417.76 ± 2.69	380.14 ± 3.03[a]	398.43 ± 2.44[ab]	364.42 ± 3.81[a]	386.75 ± 3.35[ab]	342.47 ± 2.90[a]	359.34 ± 3.36[ab]
GSSG	144.82 ± 1.75	145.16 ± 1.73	149.83 ± 1.27	168.65 ± 1.61[ab]	176.74 ± 1.85[a]	201.23 ± 2.38[ab]	199.85 ± 3.21[a]	235.26 ± 1.84[ab]
Vit C	1.26 ± 0.02	1.24 ± 0.02	1.05 ± 0.02[a]	0.92 ± 0.02[ab]	0.81 ± 0.04[a]	0.67 ± 0.03[ab]	0.68 ± 0.02[a]	0.52 ± 0.02[ab]
Vit E	0.98 ± 0.03	0.97 ± 0.03	0.93 ± 0.03	0.80 ± 0.03[ab]	0.75 ± 0.02[a]	0.66 ± 0.02[ab]	0.59 ± 0.03[a]	0.48 ± 0.03[ab]
Vit A	31.51 ± 0.51	31.27 ± 0.52	28.13 ± 0.72[a]	26.13 ± 0.72[ab]	23.47 ± 0.58[a]	20.04 ± 0.49[ab]	20.71 ± 0.58[a]	16.13 ± 0.38[ab]
TAC	3.87 ± 0.05	3.88 ± 0.05	3.04 ± 0.07[a]	3.55 ± 0.08[a]	2.64 ± 0.08[a]	3.12 ± 0.09[a]	1.96 ± 0.06[a]	2.43 ± 0.09[a]

[a]$P < 0.05$ or [a]$P < 0.01$ in comparison with pre control; [b]$P < 0.05$ or [b]$P < 0.01$ comparison between pre and post.
The CAT, SOD, GSH-Px and GSH-Rd are expressed in U/mg protein, while, GSH and GSSG are expressed in μM, Vit C, E and A are expressed in mg/dl, while, TAC expressed in μmol/L.

blood glutathione peroxidase (GSH-Px) in control group was 37.89 ± 0.63 nmole NADH oxidized/min/mg protein. The GSH-Px decreased ($P < 0.01$) in all groups of anemic women when compared with controls. After treatment the activity of GSH-Px was further found reduced ($P < 0.01$) when compared with pre-treatment levels.

The levels of vitamins C, E and A in all the non-anemic women (control) were 1.26 ± 0.02 mg/dl, 0.98 ± 0.03 mg/dl and 31.51 ± 0.52 mg/dl, respectively. These parameters were decreased in all pre-treated groups of anemic women when compared with controls. After treatment these were further decreased in mild ($P < 0.01$), moderate ($P < 0.01$) and severe ($P < 0.01$) anemic women when compared with respective pre-treatment levels. The total antioxidant capacity (TAC) of non-anemic women was 3.87 ± 0.05 μmol/L. The pre-treatment mean level of TAC decreased significantly in all anemic groups viz mild (3.04 ± 0.07 μmol/L; $P < 0.01$), moderate (2.64 ± 0.08 μmol/L; $P < 0.01$) and severe (1.96 ± 0.06 μmol/L; $P < 0.01$) as compared to pre-treatment mean TAC of control group. A significant reversal in the level of TAC was observed in mild (3.55 ± 0.08 μmol/L; $P < 0.01$), moderate (3.12 ± 0.09 μmol/L; $P < 0.01$) and severe (2.43 ± 0.09 μmol/L; $P < 0.01$) anemic women after treatment with iron and folic acid.

3.3. Oxidative stress parameters

The pre and post-treatment oxidative stress parameters of all non-pregnant healthy and anemic women are summarized in Table 3. Like, blood index parameters, and antioxidant enzymes and vitamins, the pre and post-treatment mean values of all oxidative stress parameters in controls did not differed significantly ($P > 0.05$) i.e. found to be statistically the same. Table 3 shows that the pre-treatment mean levels of lipid peroxidation products like LPO, CD and LOOH in the RBC of non-pregnant non-anemic women (control) were 2.29 ± 0.04 nmole MDA/mg protein, 43.86 ± 1.37 μM and 0.73 ± 0.03 mmol/L, respectively. The pre-treatment mean levels of these parameters were significantly ($P < 0.05$ or $P < 0.01$) increased in all groups of anemic women when compared with controls. Treatment with iron and folic acid further increased ($P < 0.05$ or $P < 0.01$) the levels of LPO in mild, moderate and severe women. Furthermore, after treatment the levels of PC were also elevated in mild ($P < 0.01$), moderate ($P < 0.01$) and severe ($P < 0.01$) anemic women when compared with respective pre-treated levels.

3.4. Experienced adverse symptoms

Adverse side effects in all four treated groups from medication during the treatment were summarized in Table 4. The both placebo and iron treated groups showed comparable side effects. The adverse symptoms were evident higher in iron treated group as compared to placebo treated group. In iron treated group, adverse effects were highest in mild followed by moderate and least in severe. On comparing, the proportions of side effects among groups were found significantly ($P < 0.01$) different ($\chi^2 = 45.84$, $P = 0.0003$).

4. Discussion

The results of present study demonstrated that treatment of iron deficient anemic women with iron and folic acid for 100 days significantly improved Hb levels and recovered most of the anti-oxidant enzymes. Recovery of haemoglobin after supplementation of iron with folic acid in anemic patients may be due to the fact that iron is an essential constituent of haeme and folic acid enhances the erythropoietic mechanism.[25] The results of present study also showed that the levels of Hct, MCV, MCH, red blood cell count and plasma iron (Fe), which were found decreased in anemic women, also recovered significantly in all supplemented groups. King et al[26] had also reported that daily oral iron, given in form of ferrous sulphate (98 mg) for 56 days daily improved the iron status of

Table 3
Blood oxidative stress parameters (Mean ± SE) of pre and post-treated healthy and anemic women.

Parameters	Control ($n = 60$)		Mild ($n = 55$)		Moderate ($n = 40$)		Severe ($n = 22$)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
LPO	2.29 ± 0.04	2.33 ± 0.04	2.65 ± 0.05[a]	3.22 ± 0.06[ab]	3.15 ± 0.10[a]	3.51 ± 0.09[ab]	3.99 ± 0.06[a]	4.36 ± 0.07[ab]
PC	1.31 ± 0.03	1.29 ± 0.03	1.46 ± 0.06	1.72 ± 0.06[ab]	1.81 ± 0.08[a]	1.96 ± 0.07[ab]	2.03 ± 0.09[a]	2.22 ± 0.10[ab]
CD	43.86 ± 1.38	44.03 ± 1.39	46.67 ± 1.98	55.48 ± 2.49[ab]	49.19 ± 2.76	53.16 ± 2.53[ab]	55.46 ± 2.96[a]	59.85 ± 3.09[ab]
LOOH	0.73 ± 0.03	0.75 ± 0.03	0.84 ± 0.04	1.06 ± 0.04[ab]	0.92 ± 0.04[a]	1.08 ± 0.04[ab]	1.19 ± 0.05[a]	1.36 ± 0.05[ab]

[a]$P < 0.05$ or [a]$P < 0.01$ in comparison with pre control; [b]$P < 0.05$ or [b]$P < 0.01$ comparison between pre and post.
The LPO is expressed in nmole MDA/mg protein, PC (nmole/mg protein), CD (μM) and LOOH (mmole/L).

Table 4
Number of women in the four groups who experienced side effects from medication.

Side effect	Control ($n = 60$)	Mild ($n = 55$)	Moderate ($n = 40$)	Severe ($n = 22$)
Dyspepsia	9 (15.0%)	12 (21.8%)	10 (25.0%)	4 (18.2%)
Loose motions	4 (6.7%)	5 (9.1%)	3 (7.5%)	2 (9.1%)
Constipation	5 (8.3%)	4 (7.3%)	4 (10.0%)	2 (9.1%)
Nausea and vomiting	0 (0.0%)	16 (29.1%)	0 (0.0%)	0 (0.0%)
Rash and itching	0 (0.0%)	1 (1.8%)	0 (0.0%)	0 (0.0%)
Headache or bodyache	0 (0.0%)	0 (0.0%)	4 (10.0%)	2 (9.1%)
Malaise/fever	0 (0.0%)	0 (0.0%)	3 (7.5%)	0 (0.0%)

women with low iron stores in anemia; however, the subjects were non-anemic but were iron deficient.

In the present study, we also observed significantly increased levels of RBC lipid peroxidation products (LPO, CD and LOOH) in women with iron deficiency anemia before and even after treatment with oral iron. There have been reports that lipid peroxidation of RBC was significantly accelerated in iron deficiency anemia.[27,28] It is well known that lipid peroxidation is a free-radical-mediated phenomenon and that the lipids in RBCs are susceptible to peroxidation in the pathophysiology of iron deficiency anemia. There are reports that the iron doses used for correcting iron deficiency anemia may further elevate the lipid peroxidation products, mainly due to increased bioavailability of elemental free iron in gastrointestinal mucosal cells of the subjects.[29,30] Moreover, reports increased peroxidative damage of RBC membrane proteins, as measured by the protein carbonyl content, in the iron supplemented groups. This may be due to Fe mediated generation of ROS, which enhance peroxidative damage of both the proteins and lipids.[31]

The activity of all antioxidant enzymes, namely CAT, SOD, GSH-Px and GSH-Rd were found decreased significantly in pre-treated anemic women, whereas their activities recovered significantly after treatment with oral iron and folic acid, except for GSH-Px. Our results are consistent with the earlier reports that CAT and SOD levels are significantly reduced in iron deficient anemic women.[32] It has been reported that an excess of reactive oxygen species, especially H_2O_2, interfere with the activity of SOD for dismutation of superoxide radical (O_2^-) via feedback mechanism.[33] Our results are similar to those of Bartal et al[34] who reported that erythrocytes in IDA are more susceptible to oxidation. The reactivation of catalase and SOD following iron and folic acid supplementation may be explained on the basis of increased availability of haeme and improvement in antioxidant status in treated women.

Interestingly we observed that the GSH-Px activity was decreased significantly in pre-treated groups and it was further reduced after treatment with iron and folic acid. Earlier Isler et al[35] also reported similar results. GSH-Px activity depends on the availability of NADPH, which is produced by pentose phosphate pathway and has been reported earlier that this pathway is impaired in iron deficiency anemia.[36] This may be a possible reason for decreased activity of GSH-Px. We also found significantly decreased erythrocyte GSH-Rd activity in pre-treated groups as compared with controls. However, following treatment, the activity of this enzyme was found recovered. Increased levels of GSSG in iron and folate treated anemic women, as observed in this study, clearly suggest that GSH in addition to GSH-Px has been utilized by some other mechanisms for neutralization of reactive oxygen species (ROS). Additionally, we also investigated total antioxidant capacity (TAC) of the anemic women and observed that TAC levels were low in iron deficient anemic women and it recovered significantly after iron and folic acid treatment. TAC considers the cumutative action of all the antioxidants present in the RBC and body fluids and provides an integrated parameters rather than the simple sum of measurable antioxidants. To our knowledge, no previous study has investigated the blood lysate TAC levels in iron deficiency anemia before and after iron and folic acid supplementation.

Interestingly, we also observed that the levels of vitamin C, E and A were decreased significantly in iron deficient anemic women. The decrease in endogenous ascorbic acid may be due to its extensive use as antioxidant to protect the gastrointestinal tract from the free radical damage during repletion of iron as well as to reduce ferric iron into ferrous iron to facilitate increased absorption of iron.[37] Furthermore, decreased vitamin C content following increased levels of lipid peroxidation products following oral iron supplementation, as observed by us in anemic women, may also be due to its utilization. The same may be true in case of vitamin E, as serum levels of this vitamin in anemic women were also found decreased both pre-treatment as well as post-treatment. Vitamin E is a vital lipid-soluble antioxidant. It is a chain-breaking antioxidant involved in the inhibition of propagation of free-radicals.[38] Our results are in agreement with to previous reports, which found decreased vitamin A in iron deficiency anemia.[39] Vitamin A deficiency is a remarkable draw back, as observed by us during iron supplementation in anemia, as it may further aggravate anemia by impairing the differentiation and proliferation of pluripotent haematopoietic cells, disturbing renal and hepatic erythropoietin synthesis, reducing mobilization of body iron stores and also disturbing iron and haeme metabolism.[40,41]

Iron deficiency is the most common nutritional deficiency worldwide, affecting approximately two billion people, mostly women and children, the prevalence of worm infestations were found to be very high. Sharma et al[42] reported that helminthes and protozoan intestinal infestations is an important cause for anemia in pregnant women in India and also suggested deworming treatment with oral iron supplementation is effective. Casey et al[43] reported that a weekly iron–folic acid supplementation and regular deworming program is associated with improved haemoglobin and iron status indicators when made available to Vietnamese women over a 12 months period. It may be pointed out that in such studies, it would have been interesting to have data on the type and magnitude of intestinal parasitic infection. Unfortunately, even in the present study, the effect of deworming cannot be assessed.

It is well known that side effects of oral iron supplementation on gastrointestinal tract may be troublesome and are one of the causes of non-compliance. Different iron salts are available to counter this problem but they decrease the availability of elemental iron for absorption. This aspect of oral iron has not been adequately addressed at both national and international levels.

On the basis of our results, it may be safely concluded that although recommended oral iron dose has good efficacy for Hb improvement in anemic women, however, the problem is that of the associated compartment of oxidative stress. Moreover, we found that in the mild anemic group less iron is utilized, which leads to make available more free iron in the body which in term may eventually further enhance the oxidative stress levels in this group. Our results clearly demonstrate higher oxidative stress in mild anemic group of women after treatment. Therefore, it may be suggested that the doses of oral iron may be given according to individual requirements, which may not only help in increasing the Hb levels but also ultimately decrease the oxidative stress components in treated groups. However, more studies, with bigger sample size, are needed to further investigate this problem.

Statement of authorship

The specific contribution of each author to the work: A.K.M. Tiwari – sample collection, writing of the manuscript, experimental work and results; A.A. Mahdi – idea, concept, study design, planning and reviewing the manuscript; F. Zahra – patient recruitment; S. Chandyan, M.M. Godbole, S.P. Jaiswar and V.K. Srivastava – estimation of blood profile, antioxidant enzymes and vitamins, oxidative stress parameters, and observed side effects from medication; M.P.S. Negi – statistical analysis. All authors also contributed their part in rectifying the valuable reviewer's comments.

Conflict of interest statement

Nil.

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