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Effect of Iron and Folate Supplementation on Pb levels in Pregnant Anemic Women: A Prospective Study

Running title: Iron Supplementation vs. Lead Levels in Pregnant Anemic Women

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Abstract

Background: There are few reports revealing association between iron intake and environmental lead exposure during pregnancy. Therefore, the present study was undertaken to investigate the effect of iron supplementation on biochemical modulation of certain lead toxicity markers associated with pregnancy.

Methods: Iron and folic acid supplementations were given to 250 pregnant anemic women (mild = 100, moderate = 100 and severe = 50) and 100 age matched non-anemic pregnant women as controls for 100 days. Lead (Pb) toxicity markers, enzymatic and non-enzymatic antioxidant were estimated as per standard protocols.

Results: The levels of Pb, serum transferrin receptors (sTfR), zinc protoporphyrin (ZPP), δ-aminolevulinic acid (δ-ALA, both in blood and urine) were found significantly increased in all pre treated subjects and these were decreased after oral iron supplementation. Iron deficient pregnant women reflected a significant increase in lipid peroxide levels (LPO) and protein carbonyl levels (PC) which were found to be further increased after iron supplementation. The levels of iron (Fe), haemoglobin (Hb), ferritin, delta aminolevulinic acid dehydratase (δ-ALAD), catalase (CAT), superoxide dismutase (SOD), and reduced glutathione levels (GSH) were significantly decreased in pre-treated groups and these parameters were found significantly increased in all supplemented subjects after treatment. Antioxidant vitamins *viz.* C and E were found significantly decreased in all post treated groups.

Conclusion: Our observation suggests that recommended iron dose is not only effective for blood indices parameters but it also decreases Pb concentrations in the blood during pregnancy. However, further studies with larger sample size are needed to confirm these findings.

Key Words: Oxidative stress, Iron Supplementation, Lead levels, Pregnancy, Iron deficiency anemia

Introduction

Lead is one of the richest heavy metals present in the earth's crust and broadly distributed and mobilized in the environment (1). Human exposure to lead occurs through various sources like lead based paints, battery recycling, lead containing pipes or lead-based solder in water supply systems, industrial processes such as lead smelting and coal combustion, grids and bearings, etc. Although lead toxicity is widely reported yet, complete control and prevention over lead exposure is still far from being achieved (2). Lead is concerned to a broad range of physiological, biochemical and behavioral malfunctions (3). There are certain reports that lead initiates oxidative damage in heart, kidney, reproductive organs, brain and erythrocytes (4). Furthermore, lead is known to have toxic effects on structure and functions of biological membrane. It has been observed that erythrocyte membranes are more susceptible to lead mediated damage as erythrocytes have high affinity for this metal (5). Elevated lead levels have been linked with anemia decreased IQ, impaired attention and speech performance and hypertension (6).

Although lead toxicity in children and adults is well recognized, exposure to lead is of special concern during pregnancy. Lead absorbed by the pregnant woman is readily transferred to the developing fetus (7). There is evidence from animal studies that intrauterine exposure to lead may disturb endocrine balance during pregnancy (8), and lead to abnormalities of renal structure and function (9), abnormalities of the reproductive system (10), and neuro-developmental toxicity (11) in offsprings. Human evidence corroborates these observations,

correlating prenatal exposure to lead with reduced birth weight and preterm delivery (12) and with neurodevelopmental abnormalities in offsprings (13). These concerns are especially significant for women and children in developing countries. Not only is exposure to lead common, but the toxicity of lead for pregnant women and their offspring may be amplified by nutritional deficiency and concomitant toxic exposures which often occur in poor nations (14).

There have been reports that nutrition plays a pivotal role in lead toxicity process. Nutrient factors, such as calcium, iron, zinc, phosphorous and proteins and personal factors including sex, age and genetic susceptibility can modify lead toxicity. Nutrient interactions with lead have provided evidence that deficiency of nutrients enhances lead absorption and its toxicity (15). Also, the effect of lead toxicity on iron metabolism has been explored for several decades as iron is an essential element and plays a vital role in the heme synthetic pathway. It has been noticed that more lead is absorbed from the gastrointestinal tract and it causes more toxic effects in case of iron deficient animals as well as human subjects (16). The discovery of iron binding protein in human duodenal mucosa, which competitively binds to lead, facilitates lead-iron interaction (17).

There are very few reports revealing association between iron intake and environmental lead exposure during pregnancy. Therefore, the present study was undertaken to investigate the effect of iron supplementation on biochemical modulation of certain lead toxicity markers associated with pregnancy.

Methods

Subjects

Informed consent was obtained from each subject and the study was approved by the Institutional Ethical Committee of King George's Medical University, Lucknow, India. The present study comprised of 250 pregnant anemic women [viz. mild (100), moderate (100) and severe (50)] aged between 20-40 years and 100 aged matched pregnant healthy women as control Hb as > 11 g/dl. The subjects were selected amongst those attending the Department of Obstetrics and Gynecology, Queen Mary's Hospital, King George's Medical University, Lucknow, U.P., India. Selected subjects were all consumers of normal mixed food, not taking any drug for preceding one month, which is a part of antenatal care. The inclusion criteria of anemic subjects were according to World Health Organization (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 (18). Care was taken to ensure that all the study subjects belonged to same middle socioeconomic class (who were able to meet the basic necessities of life and same requirements of comfort), with similar food habit and not taking any drugs preceding one month of the admission. Authors excluded women were having Hb less than 6.5 g/dl, no Hb rise by 1% after three 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.

Treatment:

At recruitment, all pregnant women (non anemic and anemic) were given iron supplements (100 mg as ferrous sulphate and 500 µg folic acid; supplied by Government agency) orally once a day, for 100 days.

After three weeks of iron/folate supplementation, Hb was monitored. Those subjects, where Hb was not found 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, King George's Medical University, Lucknow. The subjects were instructed not to change their dietary or daily activities during the study. The time interval between the last iron/folate supplementation and blood collection of each subject was within one week after completion of 100 days.

Sample collection

At admission, 6 ml venous blood was taken from each subject and divided into three aliquots at the time of recruitment. 2 ml blood was transferred to a heparin containing evacuated tube and used to determine Hb, GSH, ZPP and δ-ALAD. Another 2 ml of whole blood was transferred into heparin containing tube and then centrifuged, plasma separated and used for the estimation of LPO, iron, Pb and PC while the remaining RBCs was lysed by mixing chilled water and RBC lysate was used for the estimation of CAT and SOD. Remaining 2 ml of venous blood was also centrifuged at 3000 rpm for 15 minutes, serum separated and used for the estimation of vitamin C and E.

Analytical Estimation

Blood haemoglobin was determined by using the cyanomethemoglobin method (19). The estimatation of Fe and Pb on flame atomic absorption spectrophotometer (AAS) using a direct method as described by Kaneko (20). Serum ferritin and serum transferrin receptor levels were determined by enzyme-linked immunosorbent assay kits (Spectro Ferritin, S-22 and TfR, TF-94, Ramco Laboratories Inc, Houston, Texas, USA). Blood δ-ALAD activity was measured as per European standardized method (21). ZPP levels were directly measured in whole blood by

hematofluorometer (22). The GSH levels were measured by Ellman et al (23). The lipid peroxide (LPO) levels were measured by the method of Okhawa et al (24). The thiobarbituric acid reacting substances (TBARS) of the sample were estimated spectrophotometrically at 532 nm and expressed as nmole of MDA/mg protein. The protein oxidation was measured by estimating the protein carbonyl (PC) levels by the method of Liu et al (25). 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. Catalase (CAT, EC 1.11.1.6) activity was assayed as per the method of Aebi et al (26). The CAT activity was expressed as mmole 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 (27). The reaction was monitored spectrophotometrically at 560nm. 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). Ascorbic acid (vitamin C) levels were estimated as described by Beulter (28). α-Tocopherol (vitamin E) was measured by high-performance liquid chromatography (HPLC) as per the modified method of Omu et al (29). Briefly α-tocopherol acetate was pipetted into an eppendroff tube. Into this, blood serum was added and vortex mixed; hexane extract of vitamin 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 vitamin was eluted with methanol at the flow rate of 1.5 mL/min for 15 minutes. The peak heights and the curve areas of vitamin E and their acetate were measured to calculate the amount of this vitamin in blood serum in an ultraviolet detector with 292 nm filters.

Statistical Analysis

Healthy pregnant women (control) and pregnant anemic women (mild, moderate and severe) before and after treatment were compared together using one-way ANOVA analysis of variance by Neuman-Keules post hoc test between groups. A probability p-value of < 0.05 ($p < 0.05$) was considered statistically significant. The statistical analysis was performed on commercial software INSTAT 3.0, a demo version (Graph Pad Software, San Diego, CA).

Results

Anemic markers in pregnant women:

The pre and post-treatment anemic markers of all pregnant women who were healthy or anemic are summarized in **Table 1**. The Haemoglobin (Hb) level of healthy pregnant women was 12.48 ± 1.03 g/dl. On the other hand, it was found significantly decreased in all anemic conditions viz. mild ($p<0.001$), moderate ($p<0.001$) and severe ($p<0.001$). After treatment with iron and folic acid (100 mg and 500 µg/d) for 100 days, the levels of Hb were reversed significantly in control, mild, moderate and severe by $p<0.001$, $p<0.001$, $p<0.001$ and $p<0.05$, respectively when compared with its respective pre-treated groups. Similarly, iron (Fe) level in blood plasma of mild ($p<0.001$), moderate ($p<0.001$) and severe ($p<0.001$) anemic pregnant women was found decreased when compared with controls. Treatment restored the levels of Fe in control ($p<0.001$), mild ($p<0.001$), moderate ($p<0.001$) and severe ($p<0.05$) women. Ferritin levels of control group were 37.72 ± 12.96 µg/L. On the other hand, these levels were found significantly decreased in different groups of pregnant anemic women, i.e. mild ($p<0.001$), moderate ($p<0.001$) and severe ($p<0.001$) when compared with control. After treatment with elemental iron and folic acid per day for 100 days showed a significant reversal in control, mild, moderate and severe by ($p<0.001$), ($p<0.001$), ($p<0.001$) and ($p<0.05$), respectively when compared with its respective pre-treated groups. We also observed sTfR level in blood serum of control group was 3.69 ± 1.63 mg/L. However, this level was found significantly increased in different groups of pregnant anemic women, such as mild ($p<0.01$), moderate ($p<0.001$) and severe ($p<0.001$). Treatment suppressed the level of sTfR in post treated control ($p<0.001$), mild ($p<0.001$) moderate ($p<0.001$) and severe ($p<0.01$) pregnant women.

Effects of iron and folate treatment on lead toxicity markers in pregnant women:

Table 2 summarizes blood lead level and markers of lead toxicity (δ-ALAD, ZPP and ALA) of four groups. Levels of lead (Pb) in the blood plasma of pregnant non-anemic women were 2.12 ± 0.98 µg/dl. This parameter was increased non-significantly in mild (2.31 ± 1.03 µg/dl; $p>0.05$) and significantly in moderate (2.96 ± 1.16 µg/dl; $p<0.001$) and severe anemic women (4.08 ± 1.25 µg/dl; $p<0.001$). A significant reversal in the levels of Pb were observed in control (1.67 ± 0.84 µg/dl; $p<0.01$), mild (1.83 ± 0.91 µg/dl; $p<0.01$), moderate (2.54 ± 1.07 µg/dl; $p<0.001$) and severe (3.51 ± 1.27 µg/dl; $p<0.01$) non-anemic and anemic women after treatment with iron and folic acid. Authors also observed ZPP level of control group was 3.12 ± 1.10 mg/g Hb. However, this level was found significantly increased in different groups of pregnant anemic women, such as mild ($p<0.001$), moderate ($p<0.001$) and severe ($p<0.001$). Treatment reversed the level of ZPP significantly in post treated control ($p<0.01$), mild ($p<0.01$) moderate ($p<0.01$) and non-significantly in severe ($p>0.05$) pregnant women. δ-ALAD levels of control group were 15.36 ± 2.98 U/l. On the other hand, these levels were found significantly decreased in different groups of pregnant anemic women, i.e. mild ($p<0.05$), moderate ($p<0.001$) and severe ($p<0.001$) when compared with control. After treatment with elemental iron and folic acid per day for 100 days showed a significant reversal in control, mild, moderate and severe by ($p<0.001$), ($p<0.01$), ($p<0.01$) and ($p<0.01$), respectively when compared with its respective pre-treated groups. The levels of ALA in blood and urine of control were 3.83 ± 1.49 µg/l and 0.313 ± 0.06 mg/g cr (creatinine), respectively. These levels were significantly increased in all groups of anemic women compared with controls. Treatment with iron and folic acid recovered the levels of ALA in blood significantly in control ($P<0.01$) mild ($P<0.01$), moderate ($P<0.01$) and non-significantly in severe women ($P>0.05$) compared with pre-treated levels. Similarly, treatment

also recovered the levels of ALA in urine significantly in post treated control ($p<0.01$), mild ($p<0.01$) moderate ($p<0.05$) and non-significantly in severe ($p>0.05$) pregnant women.

Effect of iron and folic acid treatment on oxidative stress and antioxidant parameters in pregnant women:

The pre and post-treatment oxidative stress parameters of all pregnant healthy and anemic women are summarized in **Table 3**. The levels of lipid peroxidationproducts like LPO and PC in the RBC of pregnant non-anemic women (control) were 2.25 ± 0.33 nmole MDA/mg protein and 1.31 ± 0.0.28 nmole/mg protein, respectively. These parameters were significantly increased ($p<0.01$ and $p<0.001$) in all groups of women when compared with controls. Treatment with iron and folic acid further increased the levels of LPO in control, mild, moderate and severe women ($p<0.001$). Furthermore, after treatment the levels of PC were also elevated significantly in control ($P>0.05$), mild ($p<0.05$), moderate ($p<0.05$) and severe ($p<0.05$) women when compared with pre-treated levels.

Table 3 reveals that the levels of catalase in the RBC of control were 55.25 ± 7.68 nmole H_2O_2 catabolized/min/mg protein. This parameter was decreased in mild (44.99 ± 5.40 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$), moderate (40.36 ± 5.16 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$) and severe anemic women (32.95 ± 4.13 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$). A significant reversal in the level of CAT was observed in control (61.58 ± 6.70 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$), mild (49.48 ± 5.73 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$), moderate (45.52 ± 6.44 nmole H_2O_2 catabolized/min/mg protein; $p<0.001$) and severe (36.55 ± 4.09 nmole H_2O_2 catabolized/min/mg protein; $p<0.01$) non-anemic and anemic women after treatment. The levels of superoxide dismutase (SOD), and reduced glutathione (GSH) of control were 1.09 ± 0.22 U/mg protein and 411.21 ± 26.73 µM,

respectively. These levels were significantly reduced in all groups of anemic women compared with controls. After treatment with elemental iron and folic acid per day for 100 days showed a significant reversal in SOD levels in control, mild, moderate and severe by ($p<0.01$), ($p<0.01$), ($p<0.001$) and ($p<0.05$), respectively when compared with its respective pre-treated groups. Similarly, the treatment increased the levels of GSH in post treated groups *viz.* control ($p<0.01$), mild ($p<0.001$) moderate ($p<0.001$) and severe ($p<0.05$) pregnant women. We also observed that the levels of vitamins C and E in all the non-anemic women (control) were 1.21 ± 0.19 mg/dl and 0.94 ± 0.25 mg/dl, respectively. These parameters were decreased in all pre-treated groups of anemic women as compared with controls. Treatment with iron and folic acid further decreased the levels of vitamin C in control ($p<0.001$), mild ($p<0.001$), moderate ($p<0.01$) and severe women ($p<0.01$). Similarly, after treatment the levels of vitamin E were also suppressed in control ($p<0.01$), mild ($p<0.01$), moderate ($p<0.01$) and severe ($p<0.01$) women when compared with pre-treated levels. Five year age distribution and start of supplementation of 100 days of gestational week of subjects are shown in **Table 4**.

Discussion

Lead has been well documented to cause anemia through different pathways by decreasing the red blood cell survival and/or by inhibiting haem synthesis. Data of present study reveal that the levels of Hb, Fe, Ferritin were decreased in pregnant anemic women, recovering significantly in all supplemented groups. Moreover, TfR levels increased significantly in all anemic groups and reversed after treatment. Authors earlier reported that daily oral iron supplementation improved body iron in iron deficient women (35). The consistent decreases in sTfR in the supplemented groups were probably due to an increase in iron supply, or to a decrease in iron requirement while the iron stores are being replenished.

In the present study, we observed significantly increased Pb concentration in all pre treated groups and significantly reduced in all the supplemented subjects. At present decades, the interaction between Pb and Fe in the biological system has been well documented. There are reports that Fe supplementation reduces Pb body load (36, 37). It is more likely that Pb is inadvertently uptaken through pathways intended for Fe (38). Reports suggest that the divalent metal transporter 1 (DMT1) is a transporter for both Fe and Pb in the small intestine and body Pb levels have been regulated in accord with Fe status. DMT1 is elevated in iron deficiency and lesser in iron overload (39, 40). Therefore a possible system could exist where variations of Fe stores within the normal range do not cause a significant increase in Pb absorption, but during periods of Fe deficiency, the level of DMT1 becomes high enough to permit for increased Pb absorption. Adequate Fe intake may serve a dual function in preventing the absorption of Pb (38). First, intake of Fe lowers the number of Pb transporters in the gut since DMT1 regulation in the duodenum is sensitive to levels of Fe uptake (41, 42). Second, since DMT1 has a much higher affinity for Fe over Pb, the presence of Fe in the gut can competitively inhibit the uptake

of Pb. Fe has been shown capable of completely inhibiting Pb uptake by DMT1 (38). The biological mechanisms come into sight in our studies suggesting the protective effects of recommended oral Fe supplementation against Pb toxicity.

Authors also observed considerable reduction of delta aminolevulinic acid dehydratase (δ-ALAD) following a significant increase of zinc protoporphyrin (ZPP) and aminolevulinic acid in anemic women, but reversed after treatment (Table 2). Our study is in accordance with the earlier reports of Austrin et al. revealing 50% inhibition of delta aminolevulinic acid dehydratase activity at a blood lead level of 15μg/dl (43) and that of Sakai and Morita (44) providing a threshold value of blood lead for delta aminolevulinic acid dehydratase inhibition being extremely low (approximately 5μg/dl). Lead inhibition of ALAD is more profound and its inhibition has been used clinically to gauge the degree of lead poisoning, thus leading to an accumulation of aminolevulinic acid, detectable in the plasma and urine even at blood lead levels less than 10μg/dl. Although ALAD inhibition is first noted at blood lead levels of 10-20 μg/dl, heme biosynthesis does not decrease until the activity of ALAD is inhibited by 80-90%, at a much higher blood lead concentration of about 55 μg/dl (45). Inhibition of ferrochelatase results in increased excretion of coproporphyrin in urine and accumulation of protoporphyrin in erythrocytes (EP). Besides, inhibition of this enzyme leads to the substitution of iron by zinc in the porphyrin ring forming zinc protoporphyrin (ZPP) (46). Nevertheless, in the abundance of hemoglobin (Hb), even in serious case of lead intoxication, increased ZPP is relatively harmless probably constituting less than 1% of the total Hb production (47).

The activity of antioxidant enzymes, namely, CAT, SOD and GSH decreased significantly in pre-treated anemic women, while their activities recovered significantly after oral iron supplementation. The decreased activities of SOD may be due to targeting the sulfhydryl

groups, concomitant with replacement of zinc ions by lead serving as important co-factors for these antioxidant enzymes inactivating them (48). Besides, lead inactivates glutathione (meant for protection of cells against free radicals) by binding to sulfhydryl groups. Consequently, synthesis of GSH from cysteine via the γ-glutamyl cycle occurs, which is usually not effective in replenishing the supply of GSH (49).

We also found significantly increased level of LPO and PC in iron deficient anemic women and even after treated subjects. There are reports on significant acceleration of RBC's lipid peroxidation in IDA, reflecting the lipids in RBCs likely to be susceptible to peroxidation in the pathophysiology of IDA. Lead causes hemoglobin oxidation, directly causing RBC hemolysis most probably due to inhibition of ALAD, resulting in an increased concentration of substrate ALA in both blood and urine. These elevated ALA levels generate hydrogen peroxide and superoxide radical concomitant with interaction of oxyhemoglobin, resulting in the generation of hydroxyl radicals (50). Progression of all the above mentioned mechanisms enables cell's extreme vulnerability to oxidative stress (51), thus leading to cell death.

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 (52, 53). 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 (54).

The levels of vitamin C and E decreased significantly in iron deficient anemic women and further decreased after treatment. This may be due to its property of quenching ROS along with metal chelation making it a potential detoxifying agent for lead. The same may be true for vitamin E as its serum levels were also found to decrease in pre-treated as well as post-treated women. Vitamin E is a vital lipid-soluble and chain-breaking antioxidant, actively involved in the inhibition of propagation of free radicals generation during oral iron treatment in anemic patients (55, 35).

Conclusions:

Concluding it may be stated that iron and folic acid supplementation significantly revert the lead levels in supplemented groups. Moreover, perturbation of prooxidants and antioxidants in pregnant anemic women indicates definite oxidative stress, which may be due to Pb intoxication. As Iron deficiency anemia is associated with elevated blood lead levels and may increase lead absorption and also has an additional independent negative impact on fetal development. Therefore, it's paramount that bioavailability of lead by any means should be controlled and steps can be taken to reduce the prevalence of anemia during pregnancy, regulatory and health agencies should consider this as a priority and make more substantial efforts towards resolving this public health problem.

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Table 1. Iron deficiency markers (Mean ± SD) of pre and post-treated healthy and anaemic women.

	Control ($n = 100$)		Mild ($n = 100$)		Moderate ($n = 100$)		Severe ($n = 50$)	
Parameters	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Hb	12.48 ± 1.03	13.37 ± 1.24***	10.49 ± 0.23[a]	1 2.02 ± 0.54***	8.00 ± 0.53[a]	10.67 ± 1.11***	6.73 ± 0.22[a]	8.65 ± 0.66***
Fe	46.73 ± 4.53	49.92 ± 2.84***	36.76 ± 8.20[a]	45.79 ± 8.11***	23.97 ± 5.42[a]	2 9.47 ± 5.84***	19.64 ± 2.84[a]	24.12 ± 0.47***
Ferritin	37.72 ± 12.9652.36 ± 12.72***	29.88 ± 10.69[a]	40.78 ± 11.50***	20.46 ± 8.43[a]	27.23 ± 8.89***	11.99 ± 3.06[a]	18.83 ± 4.71***	
sTfR	3.69 ± 1.63	2.18 ± 0.90***	4.25 ± 1.80[b]	2.78 ± 1.33***	4.99 ± 1.79[a]	3.59 ± 1.39***	5.47 ± 1.76[a]	4.39 ± 1.57**

Haemoglobin (Hb) and iron (Fe) are expressed in g/dl and µg/dl. The values of ferritin and sTfR (serum transferring receptor levels) are expressed in µg/ L and mg/L, respectively.

Significance: [a]$p < 0.001$ and [b]$p < 0.01$ as compared with the pre control group.

**$p < 0.01$ and ***$p < 0.001$ as compared with the pre treatment group.

Pre = pre treatment; Post = post treatment.

Table 2. Lead toxicity markers (Mean ± SD) of pre and post-treated healthy and anaemic women.

	Control ($n = 100$)		Mild ($n = 100$)		Moderate ($n = 100$)		Severe ($n = 50$)	
Parameters	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Pb	2.12 ± 0.98	1.67 ± 0.84**	2.31 ± 1.03	1.83 ± 0.91**	2.96 ± 1.16[a]	2.54 ± 1.07**	4.08 ± 1.25[a]	3.51 ± 1.27**
ZPP	3.12 ± 1.10	1.82 ± 0.94**	7.97 ± 2.00[a]	6.70 ± 7.04**	10.57 ± 2.69[a]	9.28 ± 2.73**	13.60 ± 3.26[a]	12.30 ± 3.27
δ-ALAD	15.36 ± 2.98	16.91 ± 3.16***	14.34 ± 3.29[c]	15.59 ± 3.31**	11.45 ± 2.75[a]	12.77 ± 2.99**	8.76 ± 2.04[a]	9.98 ± 2.02**
δ-ALA (B)	3.83 ± 1.49	3.10 ± 1.39**	4.05 ± 1.56	3.33 ± 1.42**	4.47 ± 1.96[c]	3.72 ± 1.83**	5.88 ± 1.53[a]	5.30 ± 1.46
δ-ALA (U)	0.313 ± 0.06	0.275 ± 0.06**	0.348 ± 0.09[b]	0.307 ± 0.08**	0.354 ± 0.08[b]	0.321 ± 0.08*	0.425 ± 0.09[a]	0.392 ± 0.09

Lead (Pb) is expressed in µg/dl. The values of Zinc protoporphyrin (ZPP) and δ-aminolevulinic acid dehydratase (δ-ALAD) are expressed in.

mg/g Hb and U/l, respectively. δ-aminolevulinic acid in blood and urine are µg/l and mg/g creatinine,.

Significance: $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ as compared with the pre control group.

*****$p < 0.05$, ******$p < 0.01$ and *******$p < 0.001$ as compared with the pre treatment group.

Pre = pre treatment; Post = post treatment.

δ-ALA (B) = δ-aminolevulinic acid in blood.

δ-ALA (U) = δ-aminolevulinic acid in unine.

Table 3. Oxidative stress markers (Mean ± SD) of pre and post-treated healthy and anaemic women.

	Control ($n = 100$)		Mild ($n = 100$)		Moderate ($n = 100$)		Severe ($n = 50$)	
Parameters	Pre	Post	Pre	Post	Pre	Post	Pre	Post
LPO	2.25 ± 0.33	2.84 ± 0.50***	2.85 ± 0.49^{a}	3.49 ± 0.57***	3.06 ± 0.69^{a}	3.62 ± 0.89***	4.15 ± 0.99^{a}	4.84 ± 1.01***
PC	1.31 ± 0.28	1.41 ± 0.30	1.48 ± 0.46^{c}	1.59 ± 0.46*	1.87 ± 0.45^{a}	2.01 ± 0.44*	2.19 ± 0.48^{a}	2.37 ± 0.51*
CAT	55.25 ± 7.68	61.58 ± 6.70***	44.99 ± 5.40^{a}	49.48 ± 5.73***	40.36 ± 5.16^{a}	45.52 ± 6.44***	32.95 ± 4.13^{a}	36.55 ± 4.09**
SOD	1.09 ± 0.22	1.17 ± 0.23**	0.99 ± 0.18^{b}	1.07 ± 0.18**	0.92 ± 0.18^{a}	1.02 ± 0.18***	0.68 ± 0.15^{a}	0.78 ± 0.16*
GSH	411.21 ± 26.73	424.38 ± 29.02**	368.45 ± 29.63^{a}	383.76 ± 30.68***	354.21 ± 30.22^{a}	372.59 ± 30.78***	334.14 ± 15.20^{a}	347.60 ± 24.13*
Vit C	1.21 ± 0.19	1.12 ± 0.21***	1.01 ± 0.17^{a}	0.91 ± 0.18***	0.84 ± 0.19^{a}	0.76 ± 0.19**	0.66 ± 0.12^{a}	0 .55 ± 0.12**
Vit E	0.94 ± 0.25	0.85 ± 0.24**	0.85 ± 0.21^{b}	0.76 ± 0.20**	0.75 ± 0.14^{a}	0.66 ± 0.31**	0.51 ± 0.21^{a}	0.39 ± 0.10**

Lipid peroxidation (LPO) and prorein carbonyl contents are expressed in nmole MDA/mg protein and nmole/mg protein. The values of catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) are expressed in nmole H_2O_2 catabolized/min/mg protein, U/mg protein and µM, respectively. Vitamin C and E are expressed in mg/dl.

Significance: $^{a}P < 0.001$, $^{b}P < 0.01$ and $^{c}P < 0.05$ as compared with the pre control group.

*****$p < 0.05$, ******$p < 0.01$ and *******$p < 0.001$ as compared with the pre treatment group. Pre = pre treatment; Post = post treatment.

Table 4. 5-year age distribution and start of supplementation of 100 days in gestational week.

Age in years	**Control** (*n* = 100)	**Mild** (*n* = 100)	**Moderate** (*n* = 100)	**Severe** (*n* = 50)
20-25	19	23	20	09
25-30	42	38	40	21
30-35	24	28	26	14
35-40	15	11	14	06
Start of supplementation of 100 days in gestational week				
	21.6 ± 0.10	19.5 ± 0.09	22.3 ± 0.14	22.6 ± 0.11