IRON SUPPLEMENTATION IN YOUNG IRON-DEFICIENT FEMALES CAUSES GASTROINTESTINAL REDOX IMBALANCE: PROTECTIVE EFFECT OF A FERMENTED NUTRACEUTICAL

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The aim of this study was to assess whether the concomitant supplementation of certified fermented papaya preparation (FPP, ORI, Gifu, Japan) together with iron supplementation could beneficially affect lipid peroxidation either systemically and at a intraluminal gut level in women with low iron stores. Treatment compliance and iron absorption was assessed as well. Fifty-two non-pregnant, fertile, non-smokers, healthy women with iron deficiency were recruited. The women were given iron supplements (100 mg Fe/d as ferrous sulfate) to be taken daily for 12 weeks (group A). Group B patients were also supplemented with 6g/day of a FPP. A detailed life style questionnaire was administered to all subjects. Iron, ferritin, transferrin receptors (Tf R) and malondialdehyde (MDA) in plasma were measured. The RBCs lysate was used for the estimation of superoxide dismutase (SOD) and glutathione peroxidase (GPx). The total and free iron concentration as well as analysis of oxidative stress in the feces was measured. FPP-supplemented subjects showed a significantly lower degree of gastrointestinal discomfort ($p<0.05$) and abolished the iron supplementation-induced increase of MDA ($p<0.001$) and the depletion of SOD and GPx ($p<0.01$). Moreover, the nutraceutical co-administration brought about a significant reduction of gut oxidative damage and lower fecal content of either total and free iron ($p<0.05$ *vs* group A). Overall, group B showed a better TfR/ferritin ratio response ($p<0.05$ *vs* group A). While iron supplementation maintains its clinical relevance considering the prevalence of iron deficiency among females, a careful clinical evaluation and a protective nutraceutical co-administration, as our data suggest with FPP, should be considered.

Iron is an essential nutrient, playing a central role in oxygen transport and cellular energy metabolism. The importance of ensuring adequate bioavailable dietary iron stems from the severe consequences associated with iron deficiency and anemia, including developmental delays and irreversible cognitive deficits in young children, reduced immune function and resistance to infection, impaired physical work

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performance and adverse pregnancy outcomes. Scholastically speaking, iron deficiency anemia (IDA) is a consequence of depletion of body iron stores due to decreased iron uptake or increased iron loss/use. Body iron content is imposed by controlling its entrance into the body through the gastrointestinal system rather than by controlling its excretion.

Indeed, the International Anemia Consultative Group (1) recommends a daily supplementation of 60 to 120 mg during pregnancy depending also on the prevalence of anemia among women of childbearing age in different geographical areas and such recommendations exceed the iron RDA for pregnant women by 2- to 3-fold (2). Iron stores can be replenished through oral and parenteral therapy. In asymptomatic and mildly symptomatic patients with IDA, oral iron replacement therapy has been the mainstay therapy. Oral supplemental iron therapy is less expensive and demanding than the parenteral route, does not have potential severe side effects and is more easily available for most patients. Various iron salts have been used, ferrous sulfate being the most common but the use of oral iron is primarily limited by its gastrointestinal side effects that are mediated by non-absorbed iron.

Although newer preparations have been claimed to have less side effects, ferrous sulfate is still the most commonly used oral iron preparation. Accordingly, no difference in efficacy and side effect profile were found among ferrous sulfate, ferrous gluconate, and ferrous fumarate in a randomized, double blind study (3). On the other hand, it is recognized that "free iron" stimulates lipid peroxidation which leads to cell and tissue damage (4) and this has also been shown in *in vivo* animal studies (5, 6). Moreover, only a few studies have been conducted to investigate the effect of iron supplementation on oxidative stress in humans and, despite some contradictory reports (7), most available clinical data suggest a detrimental role of uncontrolled iron supplementation (8, 9).
Recently, it has been shown that recommended doses of iron effective for improving Hb cause an increase of lipid peroxidation end products when used by non-anemic women with marginal iron status deficiency (10). A certified fermented papaya-based nutraceutical (FPP, Immun-Age®, ORI, Gifu, Japan, made under ISO 9001 and ISO 14001 obtained from a patented biofermentation process of non-OGM carica papaya) has been shown to possess effective redox-modulating properties either in *in vitro*, experimental and in clinical setting, as summarized in a recent review and with stronger antioxidant properties as compared to vitamin E and commercially available multivitamin preparations (11). In particular, as far as this study concerns, it has been shown by authoritative haematological and molecular biology research groups that it could effectively exert iron-chelating properties (12, 13). The aim of this study was to determine whether the concomitant supplementation of FPP together with iron supplementation in women with low iron stores for 12 weeks could mitigate systemic lipid peroxidation and intraluminal gut production of reactive oxygen species in human feces while testing treatment compliance and iron absorption as well.

MATERIALS AND METHODS

Subjects

Fifty-two non-pregnant women, aged 24-37, participated in the study. They were non-smokers, generally healthy, with regular weights for height, with blood hemoglobin >10.0 g/dL, and plasma ferritin <12 µg/dL at screening. All participants were not taking any drugs or supplements. At the checkup, before entering the study, those with diabetes mellitus (fasting blood sugar >126 mg/dl), hypercholesterolemia (fasting total cholesterol >220 mg/dl, low-density lipoprotein cholesterol >140 mg/dl, high-density lipoprotein cholesterol <40 mg/dl or triglycerides >150 mg/dl) or arterial hypertension (>140 mmHg systolic or >90 mmHg diastolic blood pressure) based on National Heart Lung and Blood Institute clinical guidelines were excluded from the study.

Subjects served as their own controls as pre-supplementation (baseline) results were compared with values measured during and at the end of supplementation. Written approval was obtained from all subjects before the study which conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Study design - diet and lifestyle questionnaire

A detailed lifestyle questionnaire was administered to all subjects who were instructed to refrain from intense physical exercise. Moreover, the web-based version of the National Institutes of Health Diet History Questionnaire (NIH DHQ) was used to assess diet history over the past month and along the study period. The NIH DHQ is a food frequency questionnaire (FFQ) consisting of 124 food items including also portion sizes. Data indicate that

this instrument provides reasonable nutrient estimates and sufficient reliability and validity. Patients were advised not to use any vitamin supplement, fortified food or tea while maintaining their usual diet and, in particular, keeping unmodified their intake of fibres and carbohydrates.

Patients were also instructed not to take any gastric acid inhibitory agent or over the counter antacid, unless specifically prescribed by their physician and to report this to the investigators team.

Supplementation regimen

The subjects were given iron supplements (100 mg Fe/d as ferrous sulfate) to be taken daily for 12 weeks, in the afternoon 2 h after lunch (group A). Group B patients were supplemented for 4 months also with 6g/day of FPP. The supplement, in the amount of 3g/sachet, was given in the morning before breakfast and in the afternoon. Venous blood was obtained on day 1 prior to supplementation (basal values) and repeated after 6 and 12 weeks of supplementation. However, the transferrin receptors (Tf R)/serum ferritin ratio was also checked at 8 weeks. Moreover, each volunteer was asked to collect 3 fecal samples at baseline, 6 and 12 weeks according to normal bowel habit for the below mentioned analysis.

Sample collection

Six milliliters of venous blood were drawn into trace metal-free polypropylene syringes from each subject at the time of recruitment and at scheduled observation period. Plasma was obtained by centrifugation, transferred into polyethylene tubes, divided into three aliquots and stored at -70°C until analysis. Four milliliters of whole blood were also transferred into heparin containing tube and then centrifuged at 3000 rpm for 15 min. The serum was separated and used to estimate iron and total iron binding capacity by a colorimetric assay using bathophenanthroline sulfonate and magnesium carbonate, whereas ferritin was tested by electrochemiluminescent immunoassay with a cobas 6000 (Roche Diagnostics), soluble transferrin receptor (sTfR) by sandwich ELISA (R&D Systems, UK) and malondialdehyde (MDA) by gas chromatography- mass spectrometry using the method of Yeo et al. (14). The red blood cells (RBCs) were lysed by mixing chilled water and RBC lysate was used for the evaluation of antioxidant enzymes namely superoxide dismutase (SOD) and glutathione peroxidase (GPx) by spectrophotometry.

Quantification of the total and free iron concentration in stool

The total iron concentration of the feces was measured after the feces collections were freeze-dried and then ashed in silica vessels at 480°C for 48 h. Afterwards, the ash was dissolved in concentrated hydrochloric acid and the solution diluted to an appropriate volume with distilled water.

Free iron, i.e. weakly bound iron, in feces was assessed by using a slightly modified method described by Simpson et al. (15). A pre-weighed sample of fecal homogenate containing 5 g feces (wet weight) was mixed with a measured volume of water to reach a final volume of 15 mL. This sample was centrifuged for 30 min at room temperature at 6000×*g* and the supernatant was retrieved. The pellet was then washed with a 10 mL water and centrifuged while the two supernatants were pooled and the total volume documented. The iron and free iron content of the resultant solutions were assessed by atomic absorption spectrophotometry and was expressed in micrograms per gram of native stool. This allowed to examine the relationship between free radical production in feces and the iron content in the intestinal lumen.

Analysis of oxidative stress in the feces

To determine the *in vitro* production of reactive oxygen species in the morning feces, the method based on dimethyl sulfoxide-methanesulfinic acid reaction was applied with slight modification (16). Each fecal sample (1-2 g) was incubated for 5 h at 37°C in tris-buffered saline (pH 7.0) containing 5% dimethyl sulfoxide, used as a scavenger for hydroxyl radicals (0.7 mol/L), glucose (0.1%), and Na_2EDTA (50 mmol/L) at 37°C. The sample was then centrifuged at 900 × *g* for 10 min at room temperature, the supernatant removed, and the protein removed as a precipitate by lowering the pH to 1.0 for 10 min by adding 12 mol HCl/L. The pH was then returned to 7.4, the sample was centrifuged at 900 × *g* for 10 min at room temperature and the supernatant was stored at −20°C before batch analysis of the methanesulfinic acid content. Standards were prepared before each assay, using 0-75 mmol methanesulfinic acid/L in the incubation medium. The control sample contained deferoxamin at the beginning of the incubation period to prevent the catalytical conversion of superoxide to the hydroxyl radical. After the reaction was halted with 500 µL deferoxamin (15 mmol/L), methane sulfinic acid was extracted and analyzed by straight phase HPLC to determine methane sulfinic acid. All samples were measured in triplicate.

Statistical Analysis

Normality of distribution was tested with the Kolmogorov-Smirnov test. Whenever distribution was not normal, data were log-transformed prior to analysis. Data are expressed as the mean±standard error of the mean (S.E.M.) of multiple determinations from an experiment. Statistical significance was determined by Student's *t*-test when two data sets were analyzed or, alternatively, by

ANOVA followed by the appropriate post hoc test for multiple data sets with the statistical software StatView (Abacus Concepts, Calabasus, CA, USA). Non-parametric methods were used to test for differences in proportions. *p*-values of <0.05 were considered statistically significant.

RESULTS

No subject was anemic at baseline and their hemoglobin and mean cell volume values were in the lower part of normal limits. At the entry, plasma iron, transferrin saturation and plasma ferritin were low (Table I). The dietary analysis did not reveal any excesses in the intakes of antioxidants or iron that might have influenced the antioxidant status of the participants (data not shown). No significant correlations was observed between estimated dietary intake of micronutrients and indicators of iron status and of baseline lipid peroxidation. After pooling together gastrointestinal complaints (nausea, constipation, abdominal cramps, diarrhea) it appeared that compared to group A, FPP-supplemented subjects showed a significantly lower degree of discomfort (Fig. 1, $p<0.05$). Although no drop out occurred, 19% (4/21) of group A subjects had to be shifted to iron administration during meals so to better tolerate the treatment and 9.5 % (2/21) were put on alternative days administration whereas no such events occurred in FPP-supplemented group (not statistically significant due to the limitation of data).

Starting from 6 weeks of supplementation, hemoglobin, mean cell volume and indices of iron status (serum iron, plasma transferrin saturation and serum ferritin) showed a statistically significant improvement which peaked at 12-week observation ($p<0.001$ *vs* baseline, Table I). There was no difference among the two groups although iron level in group B showed a not significant trend benefit as compared with group A.

As for group A, two subjects after 6 weeks and one subject after 8 weeks of supplementation were still slightly iron depleted whereas none of the above abnormalities appeared in FPP-supplemented group (group B) (data not shown, not statistically significant). Iron supplementation caused a significant decrease of TfR ($p<0.05$ *vs* baseline)

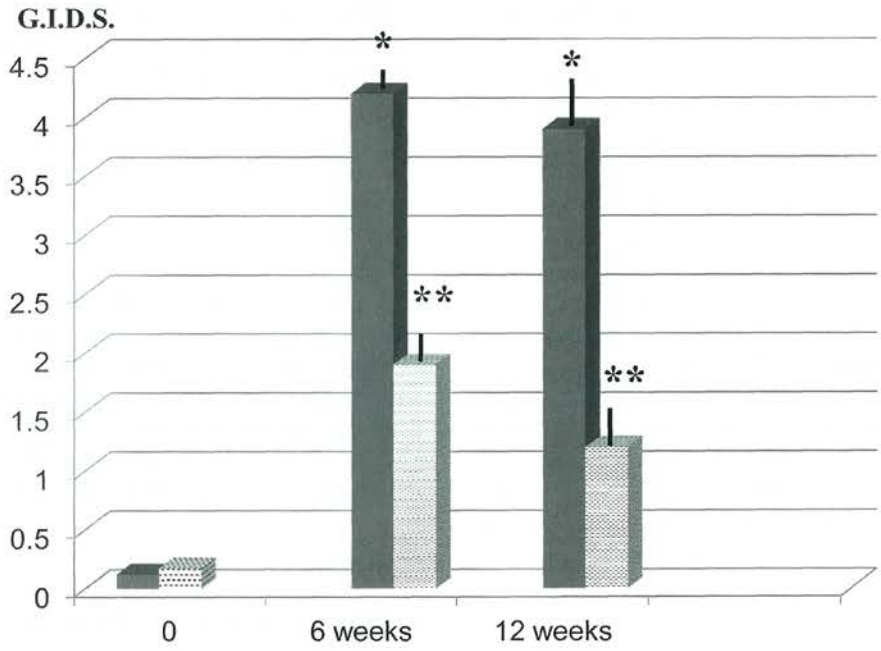

Fig. 1. *Gastointestinal discomfort during iron therapy: effect of nutraceutical supplementation with FPP. Black bars: iron supplemented subjects (group A); Dotted bars: subjects supplemented with iron and FPP (group B). G.I.D.S. (gastrointestinal discomfort score) based on nausea, diarrhea, constipation, abdominal cramps, each graded 0 to 3 (1: light, 2: mild-moderate; 3: severe) *p<0.05.*

Table I. *Baseline values of relevant biochemistry examinations: effect of iron and iron plus FPP supplementation.*

	Group A		Group B	
	baseline	12 weeks	baseline	12 weeks
Hemoglobin (g/dL)	11.3 ± 0.3	12.6 ± 0.4	11.7 ± 0.6	13.4 ± 0.7
Mean red cell volume (*fL*)	74.8 ± 0.6	89.8 ± 0.7*	77.5 ± 0.5	92.3 ± 0.4*
Plasma Iron (µg/dL)	66.8 ± 12,3	104.8 ± 17.8*	71.2 ± 10.6	126.6 ± 21.3*
TIBC (µg/dL)	354 ± 73	394 ± 62	377 ± 56	389 ± 48
Ferritin (µg/dl)	9.8 ± 2.6	18.9 ± 3.7*	9.2 ± 4.3	21.6 ± 3.8*
sTfR(µg/ml)	3.8 ± 0.9	3.4 ± 1.1*	3.9 ± 0.8	3.3 ± 0.7*
Tr Saturation (%)	16.4 ± 2.7	26.9 ± 3.4*	15.7 ± 3.6	28.1 ± 3.4*
RBC SOD (U/L)	23.3 ± 4.4	16.1 ± 3,5*	21,9 ± 2,8	20.6 ± 4.2**
RBC GPx (U/g Hb)	34.7 ± 8,1	21.2 ± 4,4*	36.1 ± 7,3	31.3 ± 8,1**

*TIBP: plasma total iron-binding capacity; sTfR: Plasma transferrin receptors; Tr Saturation: plasma transferrin saturation; RBC: red blood cells. *p<0.01 vs baseline value; **p<0.05 vs group A.*

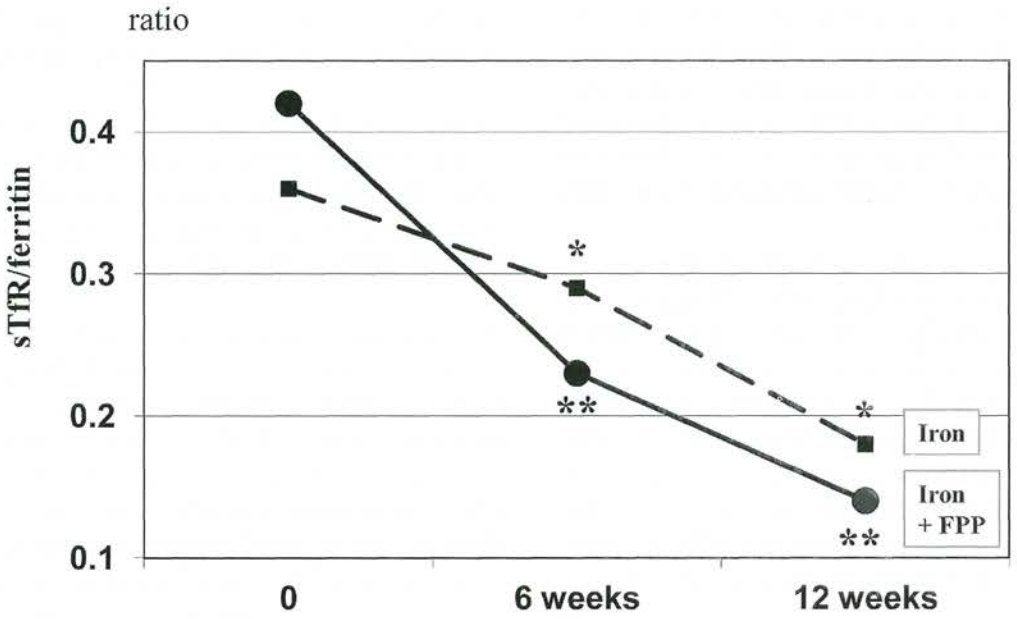

Fig. 2. *Time-course variation of sTfR/ferritin ratio during iron supplementation: effect of concomitant administration of FPP Squared indicators: iron supplemented subjects (group A); Round indicators: subjects supplemented with iron and FPP (group B). sTfR: soluble transferrin receptor. *p<0.01 vs baseline value; **p<0.05 vs group A.*

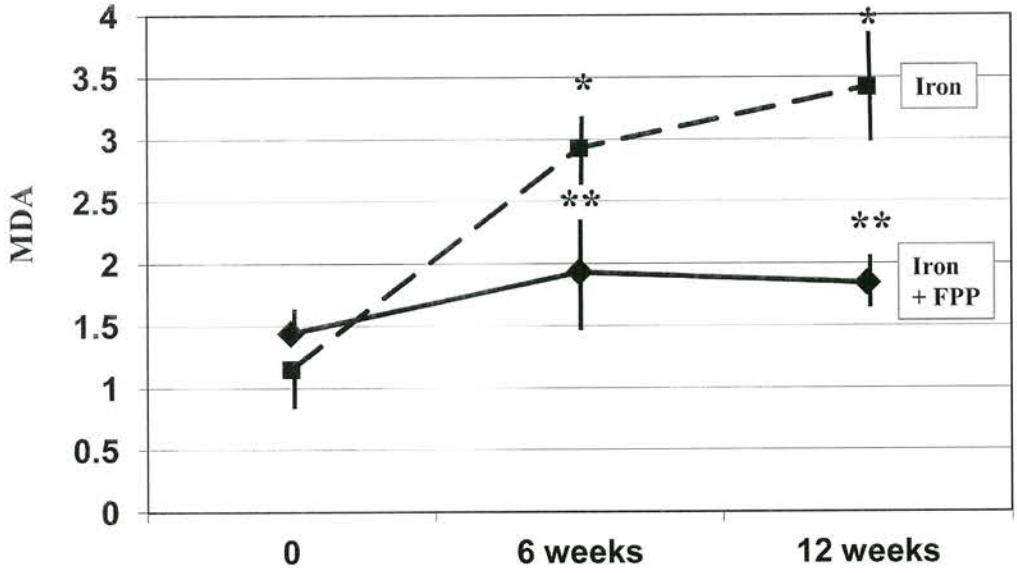

Fig. 3. *Plasma time-course MDA level in subjects administered iron therapy: effect of iron and iron plus FPP supplementation Squared indicators: iron supplemented subjects (group A); Round indicators: subjects supplemented with iron and FPP (group B). MDA: malondialdehyde. *p<0.001 vs baseline value; **p<0.01 vs group A.*

ranging from 10 % to 12% and without significant differences between the two groups. However, when looking at the Tfr/ferritin ratio, although both groups followed a comparable dietary intake, it appeared that the decrease of plasma TfR was more significant in group B ($p<0.05$ *vs* group A, Fig. 2). These data showed a significant inverse correlation with MDA (r: 0.73, $p<0.05$).

The study of systemic oxidative stress status revealed that iron supplementation brought about an increasing imbalance or redox status with a significant time-course increase of MDA ($p<0.001$ *vs* baseline values, Fig. 3) and depletion of SOD and GPx ($p<0.01$ *vs* baseline values, Table I). This phenomenon significantly appeared already at 6 weeks and displayed a further increase at 12 weeks observation. The co-administration of FPP totally abolished such phenomena ($p<0.01$ *vs* group A).

Fecal Iron Concentration

Total fecal non-heme iron in stool samples collected prior to iron supplementation was ranging from 280 to 324 mg/g dry weight and this value significantly changed during supplementation ($p<0.001$, Fig. 4a). However, under nutraceutical co-administration it appeared a partial but significant decrease ($p<0.05$ *vs* group A). The same pattern occurred also when testing the free iron concentration (Fig. 4b) where the concomitant administration of nutraceutical confirmed to yield a significant reduction of this parameter ($p<0.05$ *vs* group A).

In vitro assay of fecal oxidative stress

The reactive oxygen species (ROS) generation in fecal samples following iron supplementation is shown in Figure 5. ROS generated in stools during iron supplementation were significantly elevated when compared to baseline values ($p<0.001$). This phenomenon occurred already starting from the 6 weeks observation period and gradually increased with time with a 12-week value further enhanced ($p<0.05$ *vs* 6-week supplementation period). Such increased generation was significantly blunted in the group co-administered FPP (group B) where values

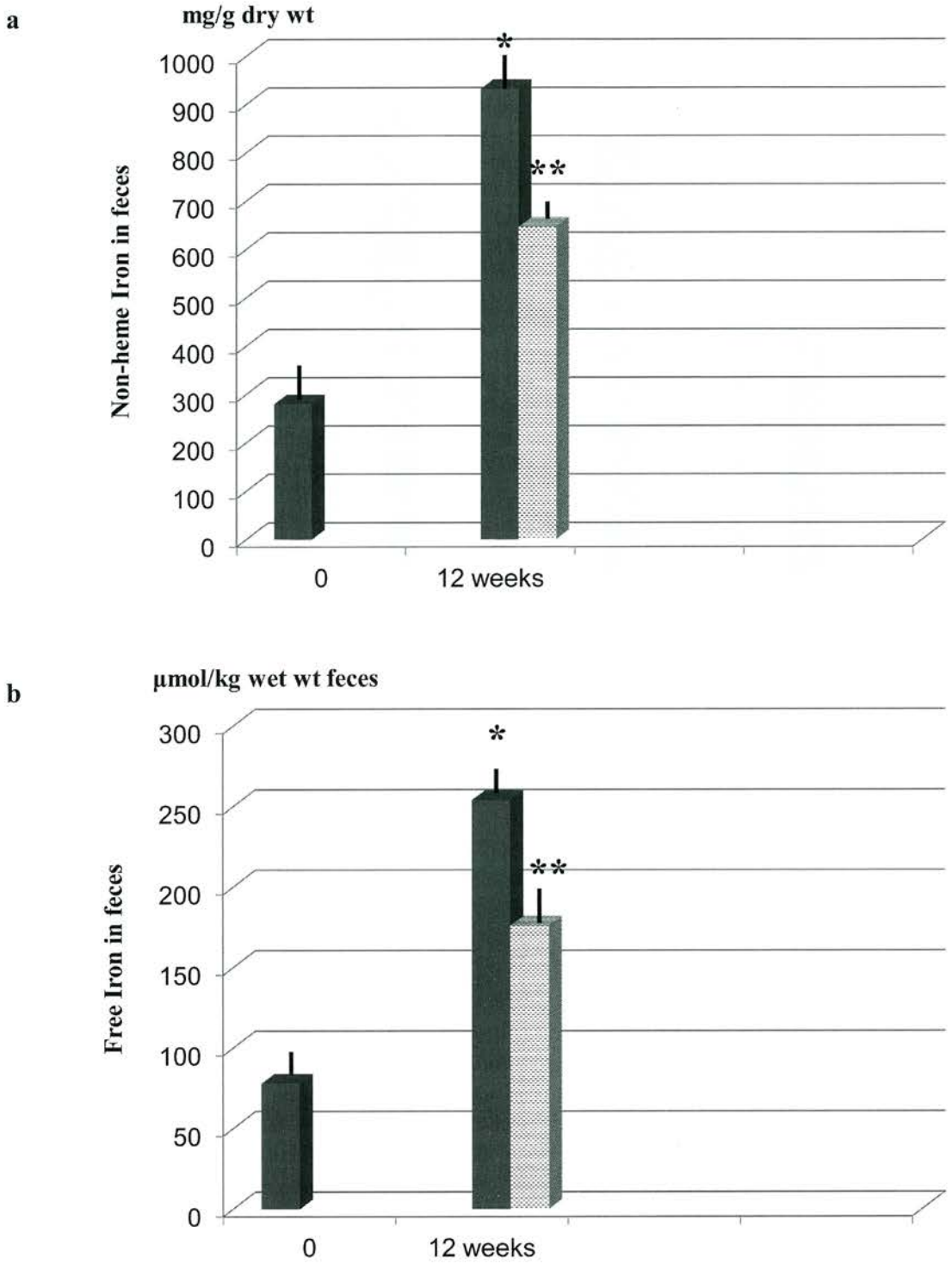

Fig. 4. *Level of non-heme iron and free iron in feces of hyposideremic females: effect of iron and iron plus FPP supplementation. Estimation of fecal level of non-heme iron (**a**) and of free iron (**b**). Black bars: iron supplemented subjects (group A); Dotted bars: subjects supplemented with iron and FPP (group B). *p<0.01 vs baseline value; **p<0.05 vs group A.*

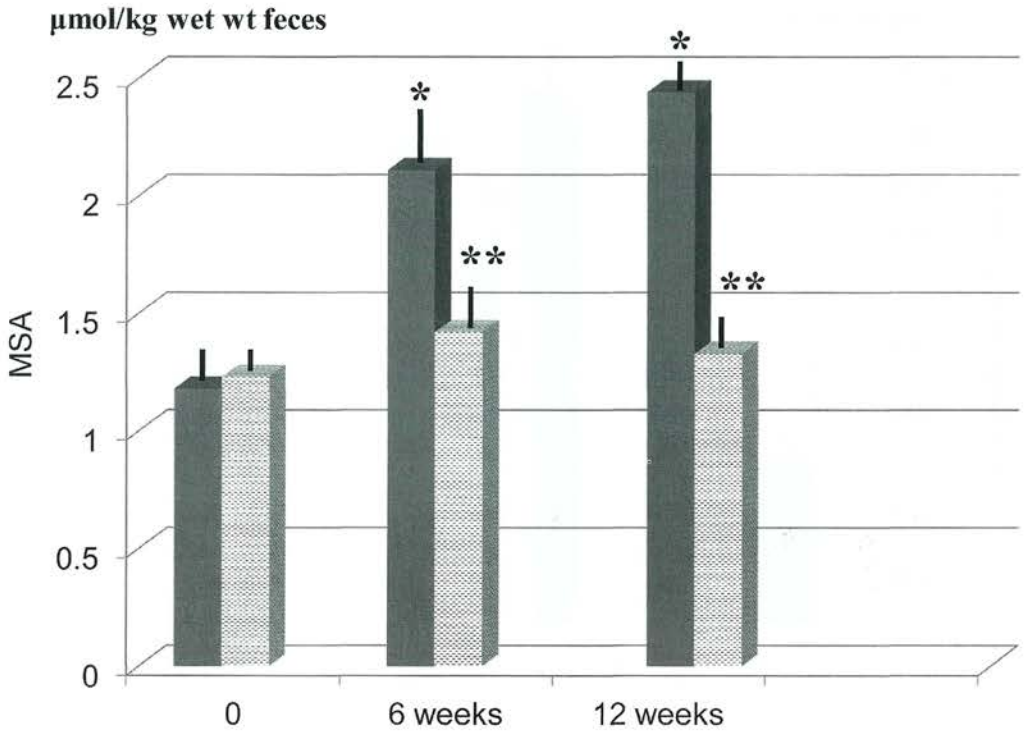

Fig. 5. *Time-course variation of in vitro Assay of Free Radical Production from feces in subjects administered iron therapy: effect of iron and iron plus FPP supplementation Black bars: iron supplemented subjects (group A); Dotted bars: subjects supplemented with iron and FPP (group B). MSA: methanesulfinic acid. *p<0.001 vs baseline value; **p<0.01 vs group A. The time-course increase of MSA level noted in A group didn't reach a statistical significance. Values for B group recorded at 6 and 12 weeks were not statistically different from baseline.*

showed a slight not-significant trend increase.

DISCUSSION

Daily iron supplementation has been traditionally a standard practice for preventing and treating anaemia and, for instance, iron supplements are generally recommended to pregnant women in most countries at doses ranging from 30-120 mg/day (1). However, its long term use may be limited by its associated adverse side effects. A large Cochrane survey has concluded that intermittent iron supplementation in menstruating women is a feasible intervention in settings where daily supplementation is not amenable for a number of reasons (17). However, in comparison with daily supplementation, the intermittent provision of iron supplements has been found to be less effective in preventing or controlling anaemia. Moreover, besides the clinically overt and easily recognizable side effects, one has also to consider possible subtly detrimental actions whose short-term impact is not known as yet but which deserves special attention in the long term. Indeed, it is known that unabsorbed dietary iron enters the colon and by interacting with intraluminal bacteria may become available for Haber-Weiss and Fenton-type reactions that generate hydrogen peroxide and hydroxyl radicals at the mucosal surface (8). Such phenomena at a mucosal level may be associated also to increased production of carcinogens and very recent epidemiological evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer through either a catalytic effect on the endogenous formation of carcinogenic N-nitroso compounds and/or the formation of cytotoxic and genotoxic aldehydes by lipoperoxidation (18).

Overall, consumption of 100 mg of daily iron as ferrous sulfate improved the iron status of women with low iron stores and at the end of the study, plasma ferritin increased by 92-117%, plasma transferrin saturation by 39-44%, hemoglobin by 10-12% and plasma level of iron normalized too. At baseline, all women were chosen to be iron deficient and as a group, these women exhibited normal levels of MDA and regular intake of dietary antioxidants and minerals. A substantial increase in markers of lipid peroxidation occurred during iron supplementation suggesting an ongoing oxidative stress. These data are consistent with previous studies in animals and humans. Although some controversies exist (7) probably explained by different iron dosages, duration of treatment and/or markers of oxidative stress used, a recent study has clearly demonstrated that supplemental oral iron intake at usual recommended doses for correcting iron deficiency may turn to be excessive as judged by elevated generation of lipid peroxidation products (19).

Indeed, supplementation with only 19 mg of iron per day as ferrous sulfate for 2 weeks has been shown to be capable to increase the concentration of weakly bound iron and the production of free-radicals in feces of adult men and women (8). It is then very likely that the repeated administration of supplemental ferrous sulfate brought about an increased free radicals formation with a vicious cycle of oxidative stress, lipid peroxidation and gut inflammation explaining why plasma MDA levels exhibited a time-course increase along the study. This finding further implies that no adaptation to the damage being caused by the daily intake of normally prescribed dosages of iron occurred. Moreover, MDA showed a significant inverse correlation with the efficiency of iron stores deposition, as judged by TfR/ferritin ration. Plasma ferritin is an acute phase protein and thus its value may not accurately reflect the iron status in the presence of infection or inflammation. It comes that the about 100% increase we observed in plasma ferritin during the study may be partly an epiphenomenon consequent to gut mucosal free radical generation with unabsorbed iron (7) and/or the systemic oxidative stress. Moreover, serum ferritin is limited in its use as a measure of the magnitude of an iron depletion because, unlike sTfR concentration, values maintain a stable level once the iron stores are exhausted (20).

The sTfR concentration is increased because more TfRs are expressed on the surfaces of cells that require additional iron. Interestingly, nutraceutical co-administration proved to yield a more significant iron stores when evaluated as TfR/ferritin ration, as compared to iron supplementation alone. Indeed, sTfR:SF ratio has been proposed as a specific evaluation of the complete distribution of iron-status and it has been found to have a good agreement between the estimated prevalence of iron deficiency and the previously described multiple indicator index in preschool children and women of childbearing age by using samples from the US NHANES 2003-2006 (21). Thus the slightly higher concentration of iron status parameters in this group, although some values didn't reach a statistical significance, together with the significantly reduced concentration of fecal iron may be tentatively interpreted as the result of a better absorption and/or transport of iron. In conclusion, supplementation with 100 mg iron per day increased indicators of lipid peroxidation in non-anemic, iron-depleted women.

Therefore, this regimen of supplemental iron as such may provide excessive amounts of iron and appears to increase the risk of oxidative stress and this equally applies to the different iron oral formulations tested (22) while the intravenous route may elicit an acute endothelial dysfunction (23). On the other hand, we have shown that the co-administration of FPP significantly mitigated these phenomena while allowing a better compliance and iron absorption. This holds of interest when considering that the association of vitamin C to enhance the bioavailability of supplemental iron has been shown to result in uncontrolled lipid peroxidation with potential adverse effects for the mother and the fetus. (24).

Although we didn't address the psychological stress factor in the present study, it is worth mentioning that very recent experimental work has shown that iron supplements further aggravated iron deposition and oxidative stress injury to the liver induced by the stress exposure. Moreover, during stress exposure, glucose-related stress hormones are negatively affected by iron supplementation which, on its turn further worsens iron deposition and oxidative stress in the liver and brain while impairing stress adaptative mechanisms (25). Interestingly, we have recently shown that FPP might

significantly mitigate psychological stress-induced redox imbalance (30). While dietary and supplemental iron remain important interventions considering that prevalence of iron deficiency among female adolescents (26), recreational athletes (27) and military recruits (28), a judicious clinical evaluation (29) and possibly a supportive nutraceutical co-administration, as our data suggest, should be carefully considered. Finally, although we didn't investigate heme-iron absorption, we have also to consider that a large number of proteins are involved and, among them, heme-iron which is taken up by heme carrier protein and its internalisation in the cytoplasm is partly linked to heme-oxygenase. The iron released from heme passes to the basal cytoplasm and is transported across the basal membrane by ferroportin, oxidized to ferric-iron and transported in the plasma by transferrin. Given that we have shown in clinics the beneficial HO-modulating properties of FPP (30), this might represent a further positive mechanism triggered by FPP to be ascertained while broader application in gastrointestinal diseases may warrants further investigations. This is also considering that over 30% of the world's population are anaemic and in developing countries every second pregnant woman and about 40% of preschool children are estimated to be anaemic.

However, it is difficult to gather precise estimations but, regarding pregnant women, for example, the prevalence is roughly 73%, 50%, 42%, 35% and 25% in South-East Asia, Africa, Western Pacific, Americas and Europe, respectively (WHO report 1998, http://www.who.int/topics/anaemia/en/). Indeed, future trial with this nutraceutical should be specifically directed to pregnant women who carry a higher risk of miscarriage when anaemic. Gut flora investigation could provide further information in the understanding of iron absorption and interaction with nutraceutical interventions. Finally, although we didn't compare the nutraceutical intervention against higher antioxidant-rich food intake, it is unlikely that the latter, besides its questionable feasibility, is unlikely to exhibit a better performance and significant epigenomic effect (31) as shown by the nutraceutical employed (11).

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