Effect of increasing iron supplementation on blood lipids in rats†

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The effects of increasing levels of Fe on serum fatty acids, cholesterol, triacylglycerol, liver and heart were examined in male Sprague–Dawley rats fed either Fe-deficient or carbonyl Fe-supplemented diets with 35 (control), 350, 3500 and 20 000 μg Fe/g for 12 weeks. As intake of Fe increased, serum total cholesterol increased from 2·0 mmol/l in controls to 5·2 mmol/l at the highest level of Fe. Also, the total serum phospholipid fatty acids increased from 609 mg/dl in controls to 1292 mg/l at the highest level of Fe. Except for the highest dose of Fe, the ratio of saturated to unsaturated phospholipid fatty acids increased from 1·2 to 1·7. The serum total free fatty acid levels remained constant among all groups with a range from 162 to 228 mg/l, while a ratio of 0·6 to 0·8 for saturated to unsaturated fatty acids was maintained. A dose-related increase in liver non-haem Fe from 18 to 3500 μg/g correlated with increases in lipid peroxidation (r 0·87), measured by the lipid-conjugated diene assay. Oxidative changes in the liver may have resulted in alterations in sterol synthesis, leading to increased serum cholesterol levels with increases in serum phospholipids and changes in the ratios of their saturated to unsaturated fatty acids. Animals with heart damage showed myocardial degeneration and cardiomyopathy with haemosiderin in interstitial macrophages or myocardial fibres and, when these were coupled with the findings of increased non-haem Fe in the heart and lipid peroxidation in the liver, suggested that oxidative stress is involved in the pathogenesis of the lesions.

Iron: Lipids: Lipid peroxidation: Cardiotoxicity: Rats

There is substantial evidence implicating Fe-catalysed reactive oxygen species in several diseases including atherosclerosis (Witztum, 1994), cancer (Stevens *et al.* 1994) and Fe overload (Burkitt & Mason, 1991). Schwartz *et al.* (1991) suggested that oxidative modification of LDL played a role in human atherosclerosis. Williams *et al.* (1991) reported that excess Fe enhanced the oxidative modification of LDL-cholesterol contributing to atherogenesis and promoted post-ischaemic myocardial injury by increasing the susceptibility of the heart to oxygen reperfusion damage. Subsequently, Smith *et al.* (1992) reported that mature atherosclerotic lesions contained detectable Fe that could promote lipid peroxidation. The mechanisms of metal ion-dependent oxidation of LDL in the presence of superoxide ($O_2^{\bullet-}$) was examined by Lynch & Frei (1993). When LDL was exposed to hypoxanthine and xanthine oxidase with and without added Fe^{3+}-citrate, they observed that oxidation was dependent upon $O_2^{\bullet-}$ and that the system generated hydroxyl radicals ($HO^{\bullet}$).

In a study involving eleven countries with more than 1100 subjects over 40 years of age, Lauffer (1991) observed that there was a direct correlation between hepatic Fe, serum cholesterol and coronary heart disease mortality. This suggested a possible synergistic effect of Fe stores with other cardiovascular risk factors. Epidemiological observations have shown that high Fe levels are associated with an increased risk of myocardial infarction in men (Salonen *et al.* 1992), and it was suggested that Fe overload could elevate the risk of acute myocardial infarction by promoting the oxidation of LDL-cholesterol. A strong association was found between high serum ferritin concentrations and the risk for acute myocardial infarction. Since this report, several studies have been published using serum ferritin, serum Fe, total Fe-binding capacity (TIBC), or transferrin saturation as the measure of Fe status (Baer *et al.* 1994; Liao *et al.* 1994; Magnusson *et al.* 1994; Sempos *et al.* 1994; Reunanen *et al.* 1995), with inconsistent results. Ascherio *et al.* (1994) reported that haem Fe rather than total Fe

consumption was correlated directly with coronary disease risk among men. Other studies have shown that serum ferritin was the strongest indicator of the presence and progression of carotid artery disease (Kiechl *et al.* 1994, 1997). In a study of an elderly Dutch population, elevated serum ferritin concentrations were associated with increased risk of myocardial infarction (Klipstein-Grobusch *et al.* 1999). The association was most evident in current or former smokers and in subjects with diabetes, raising the question of whether ferritin may adversely affect ischaemic heart disease risk in the presence of other risk factors. It may be possible that these factors interact with elevated body Fe stores and may accelerate atherogenesis by stimulating the oxidation of LDL. Rasmussen *et al.* (2001) recently reported that increased Fe stores may play a role in the development of CHD by increasing lipoprotein oxidation. In a prospective study using a case–cohort design of a population from the Atherosclerosis Risk in Communities (ARIC) study, they found that individuals carrying the haemochromatosis gene (HFE) C282Y mutation may be at increased risk of CHD.

The present study was designed to determine whether alterations in serum lipids occur in rats fed Fe-deficient or Fe-supplemented diets and the relationship to lipid peroxidation.

Materials and methods

Animals and diets

Male weanling Sprague–Dawley rats (Blue Spruce Farms, Inc., Altamont, NY, USA) were individually housed in stainless steel cages and maintained in a temperature- and light-controlled environment. Four groups of rats were fed AIN-76A diets (Dyets, Inc., Bethlehem, PA, USA) supplemented with carbonyl Fe (ISP Technologies, Inc., Wayne, NJ, USA). No Fe was added to the diet of the fifth group. The AIN-76A diet (without added Fe) contained (g/kg) casein 200·0, DL-methionine 3·0, cornstarch 150·0, glucose 500·0, fibre Celufil 50·0, AIN-76 mineral mix (without Fe) 35·0 and AIN-76A vitamin mix 10·0 with 50·0 mg menadione/kg and 2·0 g choline bitartrate/kg. The Fe-deficient group (4 μg Fe/g) received no carbonyl Fe added to the diet and the control-group diet contained 35 μg Fe/g. Animals in the other three groups received 350, 3500 or 20 000 μg Fe/g diet (Table 1).

The rats were initially divided into five groups of approximately equal mean body weight and were given free access to food and distilled, deionized water for 12 weeks.

Table 1. Diets given to rats for 12 weeks

Group	Carbonyl iron dose (μg Fe/g)	Number of rats
1	0*	9
2	35†	10
3	350	10
4	3500	8
5	20 000	10

* Fe-deficient diet.
† Control diet.

At the end of the study, the rats were anaesthetized by intramuscular injection of 5 mg ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, NY, USA) per 100 g body weight, and decapitated. A complete necropsy was performed on each animal and tissues were fixed in 10 % neutral buffered formalin. Tissue specimens were then trimmed, processed through ascending ethanol series and toluene, embedded in paraffin and sectioned at 7 μm. One set of tissues was stained with haematoxylin and eosin (H&E) and one set with Perl's Prussian blue for Fe, which involves colourless potassium ferrocyanide reacting with ferric ions to create an insoluble blue ferric ferrocyanide (Thompson & Hunt, 1966; Luna, 1968). There was special emphasis on the liver and heart tissues for tissue weights, non-haem Fe and histopathology. The non-haem Fe content was determined by the method of Whittaker *et al.* (1996a). Other portions of the liver were processed as described further.

Lipid extraction and analysis

For lipid analysis, 0·4 ml aliquots of serum were diluted with 0·6 ml normal saline (0·9 %) and extracted in 20 ml chloroform:methanol, according to the method of Folch *et al.* (1957). Prior to the separation of the solvent phases of the lipid extract with 0·05 % calcium chloride solution, 200 μg of both ultra-pure heptadecanoic acid and L-α-phosphatidylcholine were added to each sample. Dihepta-decanoyl was utilized to correct for losses from the extraction, derivatization and chromatographic procedures. Major lipid classes were separated by silicic acid TLC in a solvent system of hexane/ethyl ether/acetic acid (80:18:2, v/v/v). Lipid bands were visualized with Rhodamine 6G and identified by comparison with authentic standards spotted in channels on either side of the same chromatoplate. Areas corresponding to phospholipids and free fatty acids were individually scraped into screw-cap tubes. Both lipid fractions were quantitatively derivatized with 15 % boron trifluoride dissolved in methanol for 2 h at 70°C, with tubes being vortexed every 15 min according to the procedure of Morrison & Smith (1964).

Fatty acid methyl esters were determined using a Hewlett-Packard 5890 II GLC equipped with a CAR-BOWAX capillary column (L: 30 m; ID: 0·25 mm; film thickness: 0·25 μm) and a flame ionization detector. Integration of spectra peaks and quantification of fatty acid methyl esters was accomplished by the Hewlett-Packard CHEM STATION software.

Lipid peroxidation

Liver tissue was assayed for lipid peroxides by the lipid-conjugated diene assay. Liver lipid-conjugated dienes were determined by a modified method of Watson *et al.* (1984). Approximately 0·5 g of tissue was homogenized for 30 s in a Polytron in 4·5 ml 0·15 M-NaCl containing 0·0008 M-butylated hydroxytoluene (BHT) and 0·003 M-Na₂-EDTA. Lipids were extracted with chloroform/methanol (2:1, v/v) according to the method described by Folch *et al.* (1957). The chloroform layer was dried under a stream of O₂-free N₂, and the weight of the extracted lipid was

Table 2. Effect of increasing iron on body and organ weights, blood parameters, liver non-haem iron and lipid peroxidation

(Mean values with their standard errors)

Parameter	0 µg Fe/g (Fe deficient) Mean	SE	35 µg Fe/g (Control) Mean	SE	350 µg Fe/g Mean	SE	3500 µg Fe/g Mean	SE	20 000 µg Fe/g Mean	SE	P value
Initial body wt (g)	42	2	43	1	41	1	42	2	40	1	0·734
Final body wt (g)	286†	13	385‡	9	375‡	9	295†	9	125*	6	<0·001
Liver wt (g)	7·24*	0·27	8·83†	0·29	9·07†	0·33	9·62†	0·41	6·43*	0·33	<0·001
Liver wt/body wt (%)	2·55*	0·05	2·29*	0·03	2·41*	0·05	3·28†	0·18	5·17‡	0·18	<0·001
Liver non-haem Fe (µg/g)	17·7*	1·2	112*	5	234*	21	911†	45	3501‡	163	<0·001
Liver conjugated diene (µmol/mg lipid)	0·019*	0·001	0·019*	0·001	0·023*	0·001	0·032‡	0·002	0·039‡	0·002	<0·001
Heart wt (g)	1·51*†	0·04	1·41*	0·04	1·32*	0·05	1·73†	0·09	1·27*	0·06	<0·001
Heart wt/body wt (%)	0·54†	0·02	0·37*	0·01	0·35*	0·01	0·59†	0·04	1·03‡	0·07	<0·001
Heart non-haem Fe (g/g)	20·7*	0·4	34·4†	0·7	39·2†‡	2·0	40·5†‡	2·4	44·4†	1·1	<0·001
Serum Fe (µg/l)	200*	20	1500	130	1550*	190	980*†	360	850*†	250	<0·001
Serum TIBC (mg/l)	764†	24	489*	15	515*	23	496*	48	469*	59	<0·001

Ten rats per treatment group except for the groups receiving 0 µg Fe/g diet (n 9) and 3500 µg Fe/g diet (n 8). Means for a variable not sharing a common symbol (*, †, ‡) are significantly different (P<0·05) as determined by the Scheffé multiple comparison method, which was applied only if significant differences were determined to exist by ANOVA.

determined. The lipid was dissolved in 2 ml cyclohexane and absorbance was determined spectrophotometrically at 234 nm with cyclohexane as a blank. The lipid-conjugated diene concentration was calculated by dividing the absorbance reading by the molar absorptivity. The concentration value was expressed as µmol/mg lipid.

Statistical analysis

Differences among dietary treatment groups were assessed by one-way ANOVA, using the ABstat (1993) general linear model program. The Scheffé multiple comparison method was used to differentiate among means for variables that were significantly affected by the treatments (Snedecor & Cochran, 1980). Values are expressed as mean with their standard errors. Correlation coefficients were determined using Pearson's product moment correlation matrix (Snedecor & Cochran, 1980).

Results

Liver

After 12 weeks of treatment, hepatocellular hypertrophy, manifested as enlargement of the individual hepatocytes, was observed in six of ten animals fed diets containing 20 000 µg Fe/g. The enlarged hepatocytes, located predominantly in the periportal region of the liver lobules, were characterized by an increased amount of cytoplasm in relation to the nucleus and a large amount of granular cytoplasmic Fe. In the control group, a small amount of Fe was observed only in macrophages found in the liver sinuses. As shown in Table 2, the increase in liver non-haem Fe concentration was significantly greater in those groups of rats fed diets containing 3500 and 20 000 µg Fe/g. The increase in non-haem Fe was also paralleled by an increase in the ratio of liver weight:body weight in animals receiving Fe-fortified diets.

As the amount of Fe in the diet increased, liver conjugated dienes increased from 0·019 to 0·039 µmol/mg lipid (Table 2). Total liver non-haem Fe was directly correlated with conjugated dienes (r 0·90).

Serum iron and total iron-binding capacity

Serum Fe and TIBC of Fe-overloaded rats were not significantly different from those of control animals (Table 2). The animals receiving the Fe-deficient diet, however, had significantly decreased serum Fe levels and increased TIBC levels when compared with the control animals.

Serum lipids

Serum cholesterol increased from 2·0 for control animals to 5·2 mmol/l in animals fed the highest dose of Fe (Table 3). As the Fe dose increased there was an increase in serum cholesterol with the exception of the Fe-deficient group. Serum triacylglycerol levels did not increase with Fe dose and the only statistically significant difference found was between the Fe-deficient and 3500 µg Fe/g groups (Table 3). Serum phospholipid fatty acids generally increased with

Table 3. Effect of increasing levels of iron on serum lipids
(Mean values with their standard errors)

Parameter	0 µg Fe/g (Fe deficient)		35 µg Fe/g (Control)		350 µg Fe/g		3500 µg Fe/g		20 000 µg Fe/g		P value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Serum cholesterol (mmol/l)	2·55†	0·18	2·02*	0·11	2·54*†	0·21	3·90†‡	0·37	5·19‡	0·54	<0·001
Serum triacylglycerol (mg/dl)	91·5*	5·9	108·5*†	8·2	118·0*†	11·3	138·3†	13·7	121·1*†	11·2	0·039
Phospholipid fatty acids saturated (mg/l)	416·8*	15·1	359·8*	21·3	358·9*	34·0	560·2*†	40·2	730·6†	76·5	<0·001
Phospholipid fatty acids unsaturated (mg/l)	345·2*	12·9	249·6*	13·4	233·1*	23·0	351·4*	45·7	561·5†	45·7	<0·001
Phospholipid fatty acids total (mg/l)	761·9*	27·2	609·4*	34·1	592·0*	56·7	911·5*	75·7	1292†	119	<0·001
Free fatty acids saturated (mg/l)	69·0	6·9	75·0	4·7	78·2	7·5	67·8	5·8	107·4	41·0	0·645
Free fatty acids unsaturated (mg/l)	93·4	6·1	132·6	7·9	134·1	15·7	108·0	11·0	120·4	30·1	0·425
Free fatty acids total (mg/l)	162·4	12·2	207·5	12·2	212·3	23·2	175·8	16·7	227·7	70·7	0·708
Phospholipid fatty acids saturated/unsaturated	1·21*	0·02	1·44*†	0·04	1·54*†	0·03	1·74†	0·22	1·29*	0·07	0·002
Free fatty acids saturated/unsaturated	0·73	0·05	0·57	0·02	0·60	0·02	0·64	0·02	0·79	0·09	0·013

Nine rats per treatment group except for the groups receiving 35 and 350 µg Fe/g diet (n 8) and 3500 µg Fe/g diet (n 7). Means for a variable not sharing a common symbol (*, †, ‡) are significantly different (P<0·05) as determined by the Scheffé multiple comparison method, which was applied only if significant.

Fe dose and saturated, unsaturated and total phospholipid fatty acids were all significantly elevated ($P<0.001$) in the group of animals fed 20 000 µg Fe/g in comparison with controls. In all of the groups with the exception of the 20 000 µg Fe/g group the ratio of the saturated to unsaturated phospholipid fatty acids increased from 1·2 to 1·7, with increasing Fe dose (Table 3).

Serum free fatty acids did not increase with Fe dose. Saturated, unsaturated and total free fatty acids were not significantly different among dietary Fe doses (Table 3).

Heart

In the heart, non-haem Fe increased with dose, and the amount of Fe was significantly higher in animals receiving 20 000 µg Fe/g diet when compared to the control group (Table 2). The ratio of heart weight:body weight was significantly higher in animals receiving the 20 000 µg Fe/g diet. The incidence of severity of cardiomyopathy increased with higher dietary concentrations of Fe and was characterized by a spectrum of lesions. These included small foci of degenerated myofibres with variable numbers of mononuclear and polymorphonuclear inflammatory cells and a few fibroblasts with or without oedema, to extensive areas of degeneration or necrosis of myofibres. There were areas of fibrosis near these degenerative or necrotic lesions, as well as disruption of the ventricular or atrial walls, and thrombi formation. Seven animals, two animals on 3500 µg Fe/g and five on 20 000 µg Fe/g, died before termination of the study. Five of these seven animals had heart damage, which included Fe in the cytoplasm of the myocardial fibres, haemorrhagic necrosis, epicardial damage and clot formation.

Discussion

In the present study, feeding rats diets supplemented with carbonyl Fe resulted in dose-related increases in liver non-haem Fe and lipid peroxidation. Liver lipid peroxidation, which was measured in liver tissue by the conjugated diene assay, was highly correlated with the total hepatic non-haem Fe level (r 0·90). The liver non-haem Fe levels of 112 µg/g for the control rat group equates well with values obtained for normal human subjects of approximately 100 µg/g. In patients with genetic haemochromatosis, liver biopsies show non-haem Fe levels greater than 600 µg/g (Whittaker *et al.* 1989); this would equate to the groups receiving the two highest doses with mean levels of 911 and 3501 µg/g. There was also an enlargement of the liver and heart with increasing Fe dose when comparing the organ weights to body weight. In heart tissue of rats fed diets containing 3500 and 20 000 µg Fe/g morphological changes characteristic of cardiomyopathy were observed. As dietary intake of Fe increased, there was a dose-related increase in heart non-haem Fe, and in animals with heart damage, there was inflammation and myocyte degeneration with haemosiderin in interstitial macrophages or in myocardial fibres. Histopathology indicated hepatocellular haemosiderosis and cellular necrosis in the heart; when these were coupled with lipid peroxidation in the liver, this suggested

that oxidative stress was involved in the pathogenesis of the lesions.

A possible mechanism for tissue damage from chronic Fe overload may be the effect of free radical formation and lipid peroxidation. Whittaker *et al.* (1992) previously reported that liver lipid peroxides were significantly increased in Fe-overloaded rats. In the human body Fe turnover appears to be limited to about 10 % per year or a daily excretion of 0·03 % (Finch, 1959). By comparison, the body Fe turnover in the rat is 0·38 % per day (Finch *et al.* 1978), which makes a deleterious finding in the rat more significant.

In haemochromatosis patients, congestive heart failure is a common complication and cause of death (Powell & Isselbacher, 1987). Olson *et al.* (1987) reported that hearts obtained at autopsy from men with genetic haemochromatosis had markedly increased amounts of Fe as compared to controls. The stainable Fe was exclusively sarcoplasmic, indicating that cardiac involvement in genetic haemochromatosis represents a storage disease and not an infiltrative process.

In the present study, we also found that serum cholesterol concentration increased directly with Fe supplementation. Brunet *et al.* (1999) administered a diet with 3 % carbonyl Fe to rats for 12 weeks and found a significant increase in cholesterol and triglycerides when compared with control animals. They also assessed key enzymes for cholesterol homeostasis and found a reduction in 3-hydroxy-3-methyl-glutaryl-Co A reductase and cholesterol 7α-hydroxylase, and an enhancement of acyl-Co A–cholesterol acyltransferase activity. They reported that this may have been a result of marked membrane lipid peroxidation that brings about fluidity drop in microsomes of Fe-loaded rats. Dabbagh *et al.* (1994) also found that Fe overload in male Sprague–Dawley rats caused a significant increase in plasma cholesterol and moderately increased lipid peroxidation in the liver. The increase in plasma cholesterol was explained by a decrease in antioxidant levels in plasma and liver. Whittaker *et al.* (1996b) examined the ability of dietary antioxidants to reduce lipid peroxidation by Fe overload. Lipid peroxidation was significantly reduced ($P<0.001$) in all animals fed the antioxidants. Total serum cholesterol levels in two groups of animals receiving the diets containing high Fe (1 %) with β-carotene alone or high Fe with β-carotene plus α-tocopherol were significantly reduced ($P<0.001$) to the level of the control group.

Foretz *et al.* (1999) examined the effects of polyunsaturated fatty acids on the expression of lipogenic genes, fatty acid synthase and spot-14-protein, in cultured rat hepatocytes. They reported that the inhibitory effect of polyunsaturated fatty acids on lipogenesis-related genes linked to a cytotoxic effect due to lipid peroxidation.

From the data of this study and the others cited, it appears that lipid peroxidation and cytotoxicity are important in altering lipid metabolism, possibly by their effect on lipogenesis-related genes (Foretz *et al.* 1999) and key enzymes for cholesterol homeostasis (Brunet *et al.* 1999). Damage to cellular lipids may result in structural alterations, such as membrane fluidity and fragility, and in functional alterations. Oxidative changes in lipoproteins may result in altered lipoprotein-receptor interaction in extrahepatic target cells, leading to changes in serum lipid profiles. The oxidative change in the liver may have resulted in alterations in sterol synthesis, leading to increased serum cholesterol levels with concurrent increases in serum phospholipids and changes in the ratios of their saturated to unsaturated fatty acids.

Further investigations are required to determine the specific mechanisms of the effects of Fe supplementation on blood lipids.

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