

# Body Iron Stores Increase Hepatic and Serum Lipid in Rats fed a Standard Western Diet

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**Abstract-** The liver is the major site for lipoprotein processing and iron storage. Non-alcoholic fatty liver disease (NAFLD) is a broad spectrum of chronic liver disorder with progressive factors hypothesized to include impaired hepatic lipid metabolism and oxidative stress. Since iron produces oxidative stress, its excess may lead to lipid peroxidation and hepatocyte damage. We hypothesized that increased hepatic iron in rats fed a western diet would progress NAFLD. We determined the effect of variation in iron on plasma and hepatic lipids and oxidative stress in a rat model without pre-existing liver damage. Rats were fed liquid diets in which 35% of the energy was fat and contained low (STD-), normal (STD) or twice the normal level (STD+) of iron. The STD+ group also received parenteral iron dextran injections. After 5 weeks liver and blood were taken for analysis. Serum cholesterol and non-esterified fatty acids were increased in STD+ compared with STD group. Serum triglyceride in STD+ rats was increased compared with STD-. Histologically the STD+ group showed foci of macrovesicular lipid droplets, whereas hepatic triglyceride was decreased in STD-. Liver expression of lipid responsive gene (SREBP-2, SREBP-1c, HMG CoA reductase, 7 $\alpha$  hydroxylase, MTP1), chemokine (MCP-1) and oxidative stress marker, haemoxygenase-1 mRNA were similar in all groups. There were significant increases in hepatic malondialdehyde and hydroxyalkenal in STD+ compared with STD group. The erythrocytes of STD+ were osmotically fragile compared with the STD group. Increased liver iron affects plasma and hepatic lipids and may progress NAFLD by impairing hepatic fat metabolism.

**Keywords-** Dietary fat, iron, iron dextran, rat model, non-alcoholic fatty liver disease.

## 1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common chronic liver disorder having a broad spectrum ranging from simple steatosis to cirrhosis. Non-alcoholic steatohepatitis (NASH) belongs within this spectrum and forms the borderline between a benign condition (steatosis) and a serious/morbid condition (cirrhosis) [1, 2]. NAFLD is classified into four types with types I and II considered benign conditions the onset of which is thought to involve increased lipid delivery and its impaired utilization. Progression of the disease from type II to III involves increased oxidative stress leading to lipid peroxidation then activation of pro-inflammatory cytokines, inflammation and parenchymal tissue replacement by connective tissue.

Iron is an essential micronutrient because it binds oxygen and accepts and donates electrons, however too much iron produces oxidative stress. Lipids are sensitive to oxidative stress in the presence of oxygen and iron. The highly reactive hydroxyl radicals generated by the iron-mediated Fenton and Haber-Weiss reactions [3, 4] react with polyunsaturated fatty acids in cell membranes yielding toxic hydroperoxides and aldehydes. Lipid peroxidation causes cell injury/death by impairing cell membrane integrity [5] and damaging cell organelles [6-8].

The hepatocyte is the parenchymal cell of the liver. It stores most iron in the body [9] and functions in *de novo* lipogenesis, secretion, uptake and degradation of lipoproteins. Given this, the hepatocyte may be damaged if iron and lipids accumulate [10, 11]. Iron overload is closely associated with lipid metabolic disorders that lead to the development of NAFLD/NASH [12-14] (reviewed by the authors [15]), atherosclerosis, myocardial infarction [16, 17] and metabolic syndrome [18, 19].

Previous studies into the progression of NAFLD have used animal models with pre-existing liver damage yielding findings with limited physiological relevance [20, 21]. We recently used a rat nutritional model with fat contents comparable to Western diets. Feeding a high-fat diet (71% energy from fat) produced type I to II NAFLD compared with the rats fed a standard diet (35 % energy from fat) [22]. Interestingly, despite similar dietary iron intakes the rats fed the standard diet had low hepatic iron content compared with rats fed high fat and a stock diet of chow [23]. This suggests that in rats fed the standard diet, low hepatic iron may protect against the development of NAFLD.

In view of this we hypothesized that increased hepatic iron in rats fed a standard Western diet would progress NAFLD. The aim of this study therefore was to feed the standard diet containing normal, deficient or twice the normal level of iron plus parenteral iron to rats to determine whether hepatic iron produced fatty liver via oxidative stress and hepatic lipid peroxidation, an indication of NAFLD

progression. Plasma lipids were determined to gain insight into hepatic lipid loads and potential cardiovascular risk. These regimens were selected because Western society consumes diets high in fat and with different iron bioavailability. Typically, vegetarians usually eat diets low in iron bioavailability, while others take extra dietary iron as supplements or clinically as oral ferrous sulphate or as parenteral injections such as iron dextran (eg. InfeonR). Since enteric and parenteral iron supplements are taken up by hepatocytes and macrophages, respectively they were used in the present study [24]. In addition, iron loading of both cell types is seen in secondary iron overload disorders and Type 4 hemochromatosis making this study clinically relevant.

## 2 Methods and Materials

This study was approved by the Animal Ethics Committee, University of Western Australia and all animals received humane care. Male Sprague-Dawley rats 5 weeks of age (135 g) were purchased from the Animal Resource Centre (Murdoch WA).

### 2.1 Animals and diets

The rats were divided into 3 groups of 10 rats. They were housed under controlled conditions (24° C at constant humidity of 55% with a 12-h light: dark cycle) and given free access to food and water for the duration of the study. Diets were purchased from Dyets Inc (Bethlehem, PA, USA) in powdered form. The diets were made into the consumable liquid form each day to avoid lipid peroxidation. The rats were fed one of three standard (STD) liquid diets for 5 weeks. The three diets were isoenergetic and identical except in the iron content, which was either deficient, 0 mg/L (STD-), normal 8.8 mg/L (STD) or twice the normal level 17.5 mg/L (STD+). The STD group was considered as the control group as this received the recommended daily iron intake recommended by American Institute of Nutrition (AIN-93) [25]. The composition of the standard liquid diet has been reported previously [23].

In the liquid form the energy content of the diet consisted 35% fat (as dietary fat in the form of corn oil, olive oil and safflower oil), 18% protein and 47% carbohydrates [26]. This diet was selected because it models an average western human diet [27]. During the second week the STD+ diet group, received daily intraperitoneal injections of 10 mg iron dextran (100 mg/ml, Sigma) for 5 days to iron load macrophages

[28]. STD- and STD groups were injected with vehicle solution.

### 2.2 Euthanasia

After 5 weeks on their diets the animals were euthanized in a fed state with 0.5 ml intraperitoneal pentobarbitone sodium (Nembutal 60 mg/ml Boehringer Ingelheim Artamont NSW). Samples of blood were taken by cardiac puncture and serum and heparinized plasma removed following centrifugation. Livers were removed immediately after exsanguinations, weighed and about 100 mg of the tissue immediately frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. Samples for histopathology were fixed in formalin and the remaining liver was divided into approximately three equal parts, wrapped in cling-plastic and immediately frozen at -80°C for later analysis of lipids, iron and malondialdehyde (MDA) and 4-hydroxyalkenal levels (4-HAE).

### 2.3 Histological grading of liver

Formalin fixed and paraffin embedded liver sections were stained with haematoxylin and eosin and van Gieson's stain for collagen. Steatosis was determined by frozen section using Oil red O for lipid staining. Steatosis was graded according to the percentage of hepatocytes containing macrovesicular fat (grade 1, 0-25%; grade 2, 26-50%; grade 3, 51-75%; grade 4, 76-100%) [29].

### 2.4 Blood analysis

Osmotic fragility of erythrocytes was determined as described by Senturk et al [30]. The saline concentration causing 50 % haemolysis was determined graphically using the Graph-Pad Prism 4.0 program.

### 2.5 Biochemical analysis

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a serum multiple analyzer (Hitachi 917, Roche Diagnostics, Mannheim, Germany).

Serum lipids including total cholesterol, high density lipoprotein-cholesterol (HDL-C) and triglycerides were measured colorimetrically using commercial kits (Thermo Electron Corporation, Noble Park, Victoria, Australia).

Plasma non-esterified fatty acids (NEFAs) were measured colorimetrically at the Royal Perth Hospital by using a NEFA C reagent kit (Novachem Pty. Ltd. Victoria, Australia).

### 2.6 Liver assays

Liver samples were thawed on ice and homogenized in 1:10 dilution with deionized distilled water. Extraction and isolation of lipid to dried lipid extract was done using the Folch technique [31]. Hepatic cholesterol and triglycerides were enzymatically assayed in aliquots of the lipid extract solubilized in isopropanol as described above.

Hepatic MDA and HAE levels were assayed in another weighed liver portion. Prior to homogenization, 10  $\mu$ L 0.5M butylated hydroxy toluene in acetonitrile was added per 1 ml of homogenate to prevent oxidation [32]. Insoluble cellular components were removed by centrifugation and the cleared supernatant assayed for the MDA and HAE aldehydes. When the supernatant was reacted with 1 methyl-2-phenylindole in the presence of HCl the chromophore generated is MDA specific, whereas in the presence of methanesulfonic acid the same chromophore reports MDA and HAE aldehydes [33, 34].

## 2.7 RT-PCR on liver samples

### 2.7.1 RNA isolation and generation of cDNA

RNA for gene expression studies was isolated from liver samples. Approximately 100 mg of tissue were homogenized in 1 ml (10 volumes) of TRI Reagent (Ambion) and isolated as per the manufacturer's instructions. Quantification and purity of RNA was determined by spectrophotometry. Ambion DNA-free kit was used to remove genomic DNA from 10  $\mu$ g of RNA. cDNA was generated by reverse transcription of aliquots of DNA free RNA using avian myeloblastosis virus reverse transcriptase enzyme and oligo (dT)<sub>15</sub> primers according to the manufacturer's instructions (Promega). cDNA products were then purified by using Ultra Clean gel spin DNA purification kit (MoBio) and stored at -20°C for real-time PCR.

### 2.7.2 Real-time PCR

Semiquantitative real-time PCR was performed on a Bio-Rad thermal cycler to determine the mRNA levels. The PCR mix contained 2  $\mu$ L of cDNA, 1  $\mu$ M of the appropriate forward and reverse primers, 2 $\times$  Quantifast probe PCR Master mix (Qiagen) in a total volume of 25  $\mu$ L. PCR consisted of 50 cycles of denaturation at 94°C for 30s, annealing at T<sub>m</sub> for 30s, and extension at 72°C for 60s. The primer sequences for each target gene as well as their optimal PCR annealing temperatures are as follows: HO-1[35], forward primer 5'-GCCTGCTAGCCTGGTTCAAG-3' and reverse 5'-AGCGGTGTCTGGGATGAACTA-3' (T<sub>m</sub> 60°C), MTTP1 [36], forward primer 5'-CACACAAGTGGCCTCTCATTAAT-3' and reverse 5'-TGCCCCATCGAGAAACT-3' (T<sub>m</sub>

60°C), SREBP1c [37], forward primer 5'-GGAGCCATGGATTGCACATT-3' and reverse 5'-AGGAAGGCTTCCAGAGAGGA-3' (T<sub>m</sub> 63°C), SREBP 2 [38], forward primer 5'-CCGTAATGATGGGCCAAGAG AAA-3' and reverse 5'-AGGCCGGGGGAGACATCAGAAG-3' (T<sub>m</sub> 63°C), 7 $\alpha$  hydroxylase, forward primer 5'-CCTTTGGAGAACGGGTTGATT-3' and reverse 5'-CAGGGAGTTTGTGATGAAATGG-3' (T<sub>m</sub> 63°C), HMG CoA reductase [38], forward primer 5'-AAGGGGCGTGCAAAGACAATC-3' and reverse 5'-ATACGGCACGGAAAGAACCATAGT-3' (T<sub>m</sub> 63°C), MCP-1[39], forward primer 5'-CAGATGCAGTTAATGCCCCAC-3' and reverse 5'-AGCCGACTCATTGGGATCAT-3' (T<sub>m</sub> 63°C).

Primer specificity was confirmed from the product size by agarose gel electrophoresis and the specificity of the PCR products checked by melt curve analysis.

Relative levels of mRNA were assessed by the fold change in the transcript levels calculated relative to the first sample as described by Gentle et al [40]. An amplification efficiency value of 2 was used for all genes. Data are expressed as the fold change per unit of  $\beta$ -actin to account for any variations in reverse transcriptase efficiency or RNA loading.

## 2.8 Statistical Analyses

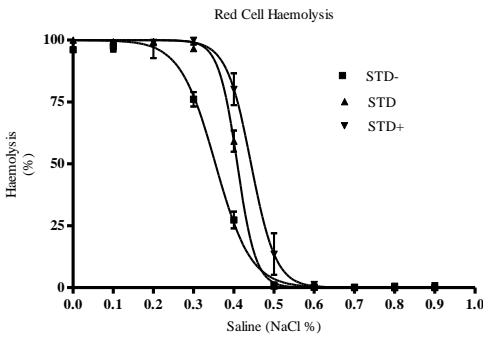
For statistical analysis InStat (Graph Pad Software, San Diego) was used for one way analysis of variance (ANOVA). Some data were not normally distributed and if the variances were not normalized by transformations, non-parametric procedures were used as indicated.  $P < 0.05$  was considered statistically significant. Results are expressed as means  $\pm$  SEM.

## 3 Results

### 3.1 Osmotic Fragility of Erythrocytes

The erythrocytes of the STD- group were least fragile being able to maintain erythrocyte membrane integrity at lower NaCl concentrations compared with the STD ( $P < 0.05$ ) and STD+ groups ( $P < 0.001$ ). By contrast erythrocytes of the STD+ group were significantly more fragile compared with erythrocytes of the normal iron group ( $P < 0.001$ ) as shown in

Fig.1.



Measurements	STD-	STD	STD+
Log EC50	0.35 ±0.003 <sup>a¶¶</sup>	0.41 ± 0.002 <sup>b¶</sup>	0.44 ± 0.006 <sup>c</sup>
Hill Slope	-9.86 ± 0.56 <sup>a§‡</sup>	-18.63 ± 3.06 <sup>b</sup>	-14.13 ± 1.79 <sup>b</sup>

Figure 1. Curve analysis of osmotic fragility curves for erythrocytes of iron deficient (STD-), normal iron (STD) and iron loaded (STD+) group. Groups bearing different superscripts are significantly different. The symbols represent statistical significance: <sup>\*</sup>p < 0.05 vs STD, <sup>¶</sup>p < 0.001 vs STD+, <sup>§</sup>p < 0.05 vs STD+ and <sup>‡</sup>p < 0.01 vs STD.

### 3.2 Liver Enzymes

There were no significant differences in the ALT levels between the different groups (Table 1). The levels of AST were significantly higher for the STD- group compared with the STD group (*P* < 0.05).

Table 1. Liver enzymes, plasma NEFAs, HDL-Cholesterol and markers for hepatic lipid peroxidation in rats fed standard diet with different iron loading

Measurements	STD-	STD	STD+
Serum ALT (U/L)	34.3 ± 1.2	33.1 ± 1.5	29.8 ± 1.5
Serum AST (U/L)	174 ± 10 <sup>a</sup>	138 ± 7 <sup>b</sup>	160 ± 7 <sup>ab*</sup>
Serum HDL-C (mmol/L)	1.0 ± 0.1 <sup>a</sup>	1.6 ± 0.1 <sup>b</sup>	1.9 ± 0.1 <sup>c¶</sup>
Plasma NEFA (mmol/L)	0.41 ± 0.02 <sup>a</sup>	0.49 ± 0.03 <sup>a</sup>	0.61 ± 0.03 <sup>b§</sup>
MDA	14.87 ±	11.55 ±	17.96 ±

(nmoles/g liver) 1.65<sup>ab</sup> 1.75<sup>a</sup> 1.00<sup>b\*\*</sup>

HAE (nmoles/g liver) 15.11 ± 2.72<sup>a</sup> 19.60 ± 2.60<sup>a</sup> 109.68 ± 5.82<sup>b§§</sup>

Groups bearing different superscripts are significantly different from each other. The symbols represent statistical significance. <sup>\*\*</sup>*P* < 0.05 by Kruskal-Wallis test, <sup>§§</sup>*P* < 0.0001 by Kruskal-Wallis test, <sup>\*</sup>*P* < 0.05, <sup>§</sup>*P* < 0.01, <sup>¶</sup>*P* < 0.0001. Data are expressed as mean ± SEM.

### 3.3 Plasma Non-esterified fatty acids (NEFAs)

Plasma NEFA concentration (Table 1) was significantly higher in the STD+ group compared with STD- and STD groups (*P* < 0.01). There was no significant difference in NEFAs between STD- and STD groups.

### 3.4 Liver Histochemistry

When the liver was stained with Oil Red O to identify lipid droplets, STD- and STD groups showed normal liver histology with no evidence of micro/macrovessicular steatosis (grade 1), whereas in the iron-loaded group (STD+ group) there were foci of pronounced periportal macrovesicular lipid droplets (zone 1 with 20% hepatocytes showing macrovesicular steatosis) (grade 1) (Fig. 2).

When the liver was stained with van Geison's to reveal collagen no increase in collagen deposition was seen in any groups.

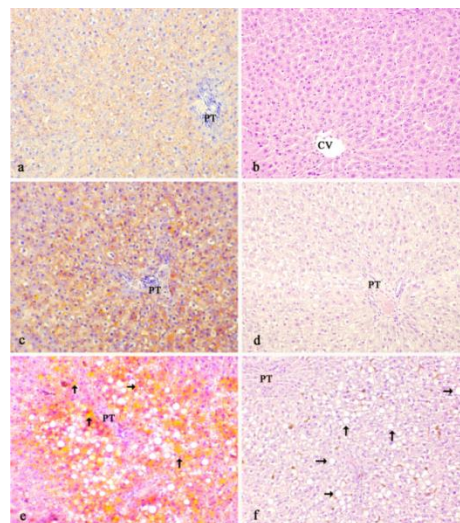


Figure 2. Hepatic histology in iron deficient (STD-), normal iron (STD) and iron-loaded (STD+) groups stained with Oil red O (a,c,e) and hematoxylin and eosin (b,d,f). Liver from an iron deficient STD- rat showing normal liver histology with minimal fat in

hepatocytes (a,b). Livers from the STD group showing normal liver architecture and normal fat deposition in hepatocytes (c,d). Iron-loaded STD+ group showing foci of periportal lipid droplets (marked by arrows) and numerous scattered iron laden macrophages (e,f). Magnification  $\times 20$ . Central vein (CV), Portal triad (PT).

### 3.5 Serum Lipids

Serum total cholesterol and High density lipoprotein-Cholesterol (HDL-C) levels were significantly different between all groups and varied directly with body iron stores ( $P < 0.0001$ ) (Fig. 3 & Table 1). Total cholesterol was decreased 29% in the STD- compared with the STD group and increased 28% in the STD+ group compared with the STD group (Fig. 3).

Similarly serum triglyceride (TG) level varied directly with body iron stores. The STD- group showed 47% less TG levels while the STD+ showed a 26% increase compared with the STD group (Fig. 3).

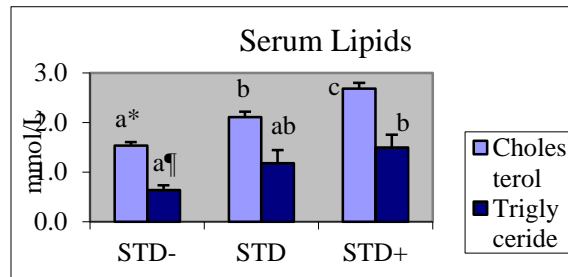


Figure 3. Serum total cholesterol and triglyceride levels in rats fed standard liquid diet with different iron loading. Iron deficient (STD-), normal iron (STD) and iron-loaded (STD+) groups. Groups bearing different superscripts are significantly different from each other. The symbols represent statistical significance: \* $P < 0.0001$ , <sup>†</sup> $P < 0.05$  after logarithmic transformation. Data are expressed as mean  $\pm$  SEM.

### 3.6 Hepatic Lipids

There was no statistical difference between the various groups in hepatic total cholesterol content (Fig. 4 & Fig. 5).

Hepatic triglyceride content was significantly lower in STD- compared with STD group and STD+ ( $P < 0.01$  after logarithmic transformation). There was no difference between STD and STD+ groups.

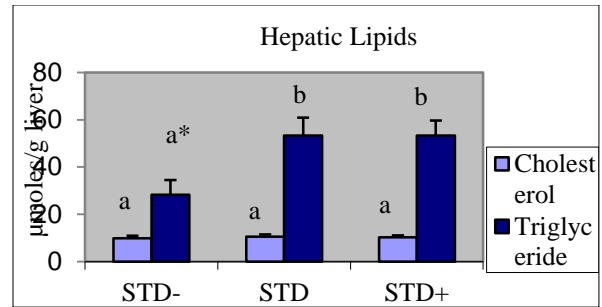


Figure 4. Hepatic cholesterol and triglyceride content in rats fed standard liquid diet with different iron loading. Iron deficient (STD-), normal iron (STD) and iron-loaded (STD+) groups. Groups bearing different superscripts are significantly different from each other. The symbols represent statistical significance: \* $P < 0.01$  after logarithmic transformation. Data are expressed as mean  $\pm$  SEM.

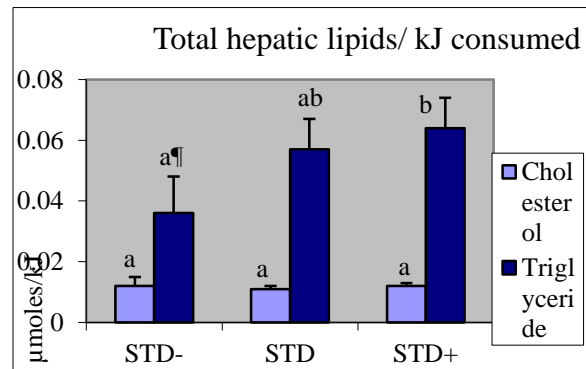


Figure 5. Total hepatic cholesterol and triglyceride content per kJ consumed in rats fed standard liquid diet with different iron loading. Iron deficient (STD-), normal iron (STD) and iron-loaded (STD+) groups. Groups bearing different superscripts are significantly different from each other. The symbols represent statistical significance: <sup>†</sup> $P < 0.05$  after logarithmic transformation. Data are expressed as mean  $\pm$  SEM.

### 3.7 Hepatic lipid peroxidation

There was no significant difference in hepatic MDA and HAE between STD- and STD groups (Table 1). In contrast hepatic MDA and HAE levels were significantly increased for the STD+ group compared with the STD group, MDA levels being 1.5 fold higher ( $P < 0.05$  by Kruskal-Wallis test) and HAE levels 5.5 fold higher for STD+ group compared with STD group ( $P < 0.0001$  by Kruskal-Wallis test).

Table 2. Relative gene expression for different groups

Genes	STD-	STD	STD+
HO-1	1.65 ± 0.55 (n=9)	1.75 ± 0.05 (n=3)	1.77 ± 0.57 (n=9)
SREBP-2	0.94 ± 0.09 (n=9)	0.86 ± 0.11 (n=6)	1.41 ± 0.25 (n=9)
SREBP-1c	0.34 ± 0.07 (n=5)	1.26 ± 0.42 (n=5)	0.82 ± 0.22 (n=5)
7 $\alpha$ hydroxylase	2.1 ± 0.9 (n=4)	2.7 ± 0.8 (n=4)	3.0 ± 1.0 (n=5)
HMG CoA reductase	1.0 ± 0.3 (n=4)	1.6 ± 0.4 (n=5)	1.3 ± 0.1 (n=5)
MTTP1	0.48 ± 0.18 (n=4)	0.93 ± 0.23 (n=4)	0.94 ± 0.12 (n=5)
MCP-1	0.53 ± 0.09 (n=5)	0.64 ± 0.20 (n=5)	0.90 ± 0.36 (n=5)

Relative change in mRNA levels in iron deficient (STD-), normal iron (STD) and iron-loaded (STD+) groups for different genes expression. The mRNA gene expression was normalized by dividing the value by the  $\beta$ -actin gene expression (house-keeping gene). Data are expressed as mean  $\pm$  SEM.

### 3.8 Gene Expression

Semiquantitative real-time PCR for different groups are shown in Table 2. Expression of  $\beta$ -actin gene was not significantly different in various groups (data not shown) validating its use as a house-keeping gene. The expressions of lipid responsive genes Sterol regulatory element binding protein-2 and -1c (SREBP-2, SREBP-1c), 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG CoA reductase), 7 $\alpha$  hydroxylase, microsomal triglyceride transfer protein 1(MTTP1) chemokine, monocyte chemoattractant protein 1 (MCP-1) and oxidative stress indicator, haem oxygenase-1 (HO-1) were not significantly different among the three groups.

### 4 Discussion

In this study rats were fed a typical diet found in Western society with iron supplementation to test the hypothesis that increased liver iron stimulates the development of NAFLD. We previously showed in these rats that despite a common starting weight the final weight of STD- rats along with their daily calorie consumption were less than the STD (control). In addition, liver iron an indication of body iron stores was decreased 2-fold in the STD- group and increased 40-fold in the STD+ group compared to the STD group [23]. Therefore, this regimen significantly altered body iron stores enabling assessment of iron on the development of NAFLD [23].

Hepatic TG content was decreased in STD- rats and can be explained by reduced calorie intake because correction for the amount of calories consumed resulted in no significant difference in TG content compared with the STD group. In contrast, the STD+ group had higher hepatic TG levels than in the STD-group even though their calorie intake was similar [23]. This suggests the extra iron stimulates lipogenesis a finding supported by Oil Red O histochemistry revealing foci of pronounced periportal macrovesicular lipid droplets in hepatocytes. This probably represents the initiation of NALFD (NAFLD Type I) in STD+ rats where the first “hit” on the liver is thought to involve aberrant lipid delivery and utilization (See also below). In contrast, other studies using methionine-choline deficient (MCD) diet or high sucrose diet to produce fatty liver combined with carbonyl iron feeding to produce hepatocyte iron overload reported reduced hepatic TG levels [20, 41]. This is proposed to be due to lipoprotein hyper-secretion and /or defective TG uptake in response to lipid peroxidation. The pre-existing liver damage produced by MCD/sucrose and hepatocyte iron overload versus normal liver coupled with iron loading of both hepatocytes and macrophages used here may explain the differences in TG content in these studies.

It is proposed that the advancement of NAFLD requires a second hit such as increased oxidative stress that stimulate cytokine expression, which in turn induces an inflammatory response that replace hepatocytes by connective tissue. We investigated hepatic oxidative stress and lipid peroxidation using the biochemical markers HAE and MDA [41-43] and found HAE elevated in STD+ rats. We hypothesized this might manifest in generalized impaired membrane function in STD+ rats by mechanisms stated in the introduction. Since erythrocytes are exposed to more oxidative stress than most other cell types due to plentiful oxygen and heme this may manifest in lipid peroxidation [44]. We tested the osmotic fragility of erythrocytes in saline and showed the erythrocytes of the STD+ group were more fragile. This is consistent with increased pro-oxidants altering membrane fluidity/stability [42]. It is known that MDA cross links erythrocyte phospholipids and exposure to oxidants increased erythrocyte membrane instability. The increased erythrocyte fragility in the STD+ rats appears moderate as there was no circulatory hemolysis as evidenced by plasma iron being exclusively transferrin-bound [23].

With respect to the liver of STD+ rats, we found no evidence of iron-induced oxidative injury as the expression of HO-1 mRNA a sensitive indicator of

oxidative stress and a key marker of the antioxidant response [42, 45] was similar in all groups. This suggests adequate buffering of reactive oxygen species by endogenous scavenger systems within the liver. Furthermore, to determine whether cytokine expression had been affected by iron we measured the expression of monocyte chemoattractant protein an important regulator of liver fibrosis [46-48]. We found no change in MCP-1 mRNA expression across the groups indicating an absence of significant liver damage and extra collagen deposition, findings corroborated by normal serum enzyme levels (ALT and AST) and Van Geison's histochemical staining for collagen, respectively. Taken together the foci of macrovesicular lipid droplets in the STD+ group represent the initiation of the first hit in NAFLD development probably due to aberrant lipid delivery and processing. In view of this we next considered serum lipid levels to gain insight into likely hepatic lipid load and cardiovascular risks.

Serum TGs varied directly with body iron stores and confirm previous reports [30, 41, 49]. Very low density lipoprotein (VLDL-TG) is the main lipoprotein that constitutes total serum TG in the fed state [50], although intestinally secreted chylomicrons (CM), and their remnants (CMR), also make contributions. It is not possible to define the site(s) at which iron affects plasma lipoproteins without profiling each lipoprotein and their site of degradation. Nonetheless, in view of the elevated NEFA levels in STD+ rats and the fact they substantially contribute to VLDL-TG formation in the fed state, we favor increased hepatic lipogenesis and hypersecretion of VLDLs in response to increased NEFA load. This would explain the increased serum TG levels, hepatic TG content and foci of lipid droplets in STD+ rats. It is unclear why iron increased NEFAs but it is not due to extra calorie intake as this was the same as STD- rats. In the fed state plasma NEFA's are derived from the peripheral lipolysis/clearance of lipoproteins, adipose cell lipolysis/release, and dietary absorption by the intestine [51] and as these sites may be affected by iron they should be considered in future work [15, 49].

Serum cholesterol concentration was directly related to iron loading. Since the diet was devoid of cholesterol, serum cholesterol is derived from endogenous sources which in the rat are mainly HDL-Cholesterol (HDL-C), but Low density lipoprotein-Cholesterol (LDL-C) and VLDL-Cholesterol (VLDL-C) contribute about 33% [52]. Serum cholesterol reflects the integration of a number of pathways involved in lipid metabolism involving its uptake, synthesis, catabolism and secretion.

Although others have shown that some of these pathways are affected by variations in iron [53-55] we saw no change in hepatic mRNA levels for genes involved with cholesterol metabolism (SREBP-2 and -1c), its synthesis (HMG CoA reductase) and its catabolism (7 $\alpha$  hydroxylase). Others have shown reduced cholesterol synthesis [53, 56] and HDL-C [53, 57], indicating a role for iron in post-transcriptional regulation of cholesterol metabolism.

It is known that LDL-C is subject to oxidation [58] and is taken up by vascular macrophages to form foam cells that contribute to atherosclerotic lesions/plaques [59, 60]. Given the high serum cholesterol coupled with a raised pro-oxidant environment of the vascular system due to iron-laden macrophages the increased erythrocyte fragility in STD+ rats indicates an increased risk to atherosclerotic lesions in this group as shown by others [16, 61].

The inclusion of lipoprotein profiling, and absorptive studies of dietary fats with variations in iron would have provided an additional insight in these mechanisms. In summary, our hypothesis that iron contributes to NAFLD is supported by showing in STD+ rats impaired hepatic fat metabolism as evidenced by isolated foci of macrovesicular lipid droplets probably due to increased hepatic lipid load but hepatic oxidative stress produced by iron appears buffered by adequate cellular antioxidants. Also in STD+ rats erythrocyte fragility indicates a risk to atherosclerosis given the high serum cholesterol and iron-laden macrophages. This regimen offers an opportunity to study the early stages in the development towards NAFLD and other risk factors.

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#### REFERENCES

- [1] Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nature reviews Gastroenterology & hepatology* 2013.
- [2] Farrell GC, Wong VW, Chitturi S. NAFLD in Asia--as common and important as in the West. *Nature reviews Gastroenterology & hepatology* 2013;10:307-18.
- [3] Moldovan L, Moldovan NI. Oxygen free radicals and redox biology of organelles. *Histochem Cell Biol* 2004;122:395-412.

- [4] Kruidenier L, Verspaget HW. Oxidative stress as a pathogenic factor in inflammatory bowel disease-radicals or ridiculous? *Alimentary Pharmacology & Therapeutics* 2002;16:1997-2015.
- [5] Ohyashiki T, Ohtsuka T, Mohri T. A change in the lipid fluidity of the porcine intestinal brush-border membranes by lipid peroxidation. Studies using pyrene and fluorescent stearic acid derivatives. *Biochim Biophys Acta* 1986;861:311-8.
- [6] Bacon BR, O'Neill R, Park CH. Iron-induced peroxidative injury to isolated rat hepatic mitochondria. *J Free Radic Biol Med* 1986;2:339-47.
- [7] Hogberg J, Bergstrand A, Jakobsson SV. Lipid Peroxidation of Rat-Liver Microsomes. Its Effect on the Microsomal Membrane and Some Membrane-Bound Microsomal Enzymes. *European Journal of Biochemistry* 1973;37:51-9.
- [8] Myers BM, Prendergast FG, Holman R, Kuntz SM, LaRusso NF. Alterations in the structure, physicochemical properties, and pH of hepatocyte lysosomes in experimental iron overload. *J Clin Invest* 1991;88:1207-15.
- [9] Andrews NC, Schmidt PJ. Iron homeostasis. *Annu Rev Physiol* 2007;69:69-85.
- [10] Havel RJ, Hamilton RL. Hepatic catabolism of remnant lipoproteins: where the action is. *Arteriosclerosis, thrombosis, and vascular biology* 2004;24:213-5.
- [11] Blasiola DA, Davis RA, Attie AD. The physiological and molecular regulation of lipoprotein assembly and secretion. *Mol Biosyst* 2007;3:608-19.
- [12] Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994;107:1103-9.
- [13] George DK, Goldwurm S, MacDonald GA, Cowley LL, Walker NI, Ward PJ, et al. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. *Gastroenterology* 1998;114:311-8.
- [14] Fargion S, Mattioli M, Fracanzani AL, Sampietro M, Tavazzi D, Fociani P, et al. Hyperferritinemia, iron overload, and multiple metabolic alterations identify patients at risk for nonalcoholic steatohepatitis. *Am J Gastroenterol* 2001;96:2448-55.
- [15] Ahmed U, Latham PS, Oates PS. Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis. *World journal of gastroenterology : WJG* 2012;18:4651-8.
- [16] Lapenna D, Pierdomenico SD, Ciofani G, Ucchino S, Neri M, Giamberardino MA, et al. Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques. *Free Radic Biol Med* 2007;42:492-8.
- [17] Tuomainen TP, Punnonen K, Nyssonen K, Salonen JT. Association between body iron stores and the risk of acute myocardial infarction in men. *Circulation* 1998;97:1461-6.
- [18] Tsimihodimos V, Gazi I, Kalaitzidis R, Elisaf M, Siamopoulos KC. Increased serum ferritin concentrations and liver enzyme activities in patients with metabolic syndrome. *Metab Syndr Relat Disord* 2006;4:196-203.
- [19] Jehn M, Clark JM, Guallar E. Serum ferritin and risk of the metabolic syndrome in U.S. adults. *Diabetes Care* 2004;27:2422-8.
- [20] Kirsch R, Sijtsema HP, Tlali M, Marais AD, Hall Pde L. Effects of iron overload in a rat nutritional model of non-alcoholic fatty liver disease. *Liver Int* 2006;26:1258-67.
- [21] Imeryuz N, Tahan Y, Sonsuz A, Eren F, Uraz S, Yuksel M, et al. Iron preloading aggravates nutritional steatohepatitis in rats by increasing apoptotic cell death. *J Hepatol* 2007;47:851-9.
- [22] Ahmed U, Redgrave TG, Oates PS. Effect of dietary fat to produce non-alcoholic fatty liver in the rat. *J Gastroenterol Hepatol* 2009;24:1463-71.
- [23] Ahmed U, Oates PS. Dietary fat level affects tissue iron levels but not the iron regulatory gene HAMP in rats. *Nutr Res* 2013;33:126-35.
- [24] Bacon BR, Tavill AS, Brittenham GM, Park CH, Recknagel RO. Hepatic lipid peroxidation in vivo in rats with chronic iron overload. *J Clin Invest* 1983;71:429-39.
- [25] Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;123:1939-51.
- [26] Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, et al. Model of nonalcoholic steatohepatitis. *Am J Clin Nutr* 2004;79:502-9.
- [27] Couzin J. Nutrition research. IOM panel weighs in on diet and health. *Science* 2002;297:1788-9.
- [28] Turbino-Ribeiro SM, Silva ME, Chianca DA, Jr., De Paula H, Cardoso LM, Colombari E, et al. Iron overload in hypercholesterolemic rats affects iron homeostasis and serum lipids but not blood pressure. *J Nutr* 2003;133:15-20.
- [29] Kirsch R, Clarkson V, Shephard EG, Marais DA, Jaffer MA, Woodburne VE, et al. Rodent nutritional model of non-alcoholic steatohepatitis: Species, strain and sex difference studies. *Journal of Gastroenterology and Hepatology* 2003;18:1272-82.
- [30] Whittaker P, Chanderbhan RF. Effect of increasing iron supplementation on blood lipids in rats. *Br J Nutr* 2001;86:587-92.
- [31] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497-509.
- [32] Botsoglou N, Fletouris D, Papageorgiou G, Vassilopoulos V, Mantis A, Trakatellis A. Rapid, sensitive and Specific Thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food and feedstuff samples. *J Agric Food Chem* 1994;42:1931-7.
- [33] Erdelmeier I, Gerard-Monnier D, Yadan JC, Chaudiere J. Reactions of N-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. *Chem Res Toxicol* 1998;11:1184-94.
- [34] Gerard-Monnier D, Erdelmeier I, Regnard K, Moze-Henry N, Yadan JC, Chaudiere J. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chem Res Toxicol* 1998;11:1176-83.
- [35] Rojo AI, Salina M, Salazar M, Takahashi S, Suske G, Calvo V, et al. Regulation of heme oxygenase-1 gene expression through the phosphatidylinositol 3-kinase/PKC-zeta pathway and Sp1. *Free Radic Biol Med* 2006;41:247-61.
- [36] Iqbal J, Dai K, Seimon T, Jungreis R, Oyadomari M, Kuriakose G, et al. IRE1beta inhibits chylomicron production by selectively degrading MTP mRNA. *Cell Metab* 2008;7:445-55.
- [37] Kim YJ, Kim KY, Kim MS, Lee JH, Lee KP, Park T. A mixture of the aqueous extract of *Garcinia cambogia*, soy peptide and L-carnitine reduces the accumulation of visceral fat mass in rats rendered obese by a high fat diet. *Genes Nutr* 2008;2:353-8.
- [38] Stangl GI, Weisse K, Dinger C, Hirche F, Brandsch C, Eder K. Homocysteine thiolactone-induced hyperhomocysteinemia does not alter concentrations of cholesterol and SREBP-2 target gene mRNAs in rats. *Exp Biol Med (Maywood)* 2007;232:81-7.
- [39] Otogawa K, Ogawa T, Shiga R, Nakatani K, Ikeda K, Nakajima Y, et al. Attenuation of acute and chronic liver injury in rats by iron-deficient diet. *Am J Physiol Regul Integr Comp Physiol* 2008;294:R311-20.
- [40] Gentle A, Anastasopoulos F, McBrien N. High-resolution semi-quantitative real-time PCR without the use of a standard curve. *Biotechniques* 2001;31:504-8.
- [41] Brunet S, Thibault L, Delvin E, Yotov W, Bendayan M, Levy E. Dietary iron overload and induced lipid peroxidation are associated with impaired plasma lipid transport and hepatic sterol metabolism in rats. *Hepatology* 1999;29:1809-17.
- [42] Otogawa K, Kinoshita K, Fujii H, Sakabe M, Shiga R, Nakatani K, et al. Erythrophagocytosis by liver macrophages (Kupffer cells) promotes oxidative stress, inflammation, and fibrosis in a rabbit model of steatohepatitis: implications for the pathogenesis of human nonalcoholic steatohepatitis. *The American journal of pathology* 2007;170:967-80.



- [43] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991;11:81-128.
- [44] Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, et al. Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood* 2003;101:5033-8.
- [45] Brown KE, Dennery PA, Ridnour LA, Fimmel CJ, Kladney RD, Brunt EM, et al. Effect of iron overload and dietary fat on indices of oxidative stress and hepatic fibrogenesis in rats. *Liver Int* 2003;23:232-42.
- [46] Imamura M, Ogawa T, Sasaguri Y, Chayama K, Ueno H. Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. *Gastroenterology* 2005;128:138-46.
- [47] Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009;50:261-74.
- [48] Mitchell C, Couton D, Couty JP, Anson M, Crain AM, Bizet V, et al. Dual role of CCR2 in the constitution and the resolution of liver fibrosis in mice. *The American journal of pathology* 2009;174:1766-75.
- [49] Kim J, Jia X, Buckett PD, Liu S, Lee CH, Wessling-Resnick M. Iron loading impairs lipoprotein lipase activity and promotes hypertriglyceridemia. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2013;27:1657-63.
- [50] Lombardi B, Pani P, Schlunk FF. Choline-deficiency fatty liver: impaired release of hepatic triglycerides. *Journal of lipid research* 1968;9:437-46.
- [51] Frayn KN, Summers LK, Fielding BA. Regulation of the plasma non-esterified fatty acid concentration in the postprandial state. *Proc Nutr Soc* 1997;56:713-21.
- [52] Tsutsumi K, Hagi A, Inoue Y. The relationship between plasma high density lipoprotein cholesterol levels and cholesteryl ester transfer protein activity in six species of healthy experimental animals. *Biological & pharmaceutical bulletin* 2001;24:579-81.
- [53] Stangl GI, Kirchgessner M. Different degrees of moderate iron deficiency modulate lipid metabolism of rats. *Lipids* 1998;33:889-95.
- [54] Rao GA, Crane RT, Larkin EC. Reduced plasma lecithin cholesterol acyl transferase activity in rats fed iron-deficient diets. *Lipids* 1983;18:673-6.
- [55] Johnston SM, Murray KP, Martin SA, Fox-Talbot K, Lipsett PA, Lillemoe KD, et al. Iron deficiency enhances cholesterol gallstone formation. *Surgery* 1997;122:354-61; discussion 61-2.
- [56] Au YP, Schilling RF. Relationship between anemia and cholesterol metabolism in 'sex-linked anemic' (gene symbol, sla) mouse. *Biochim Biophys Acta* 1986;883:242-6.
- [57] Honda T, Honda K, Kokubun C, Nishimura T, Hasegawa M, Nishida A, et al. Time-course changes of hematology and clinical chemistry values in pregnant rats. *J Toxicol Sci* 2008;33:375-80.
- [58] Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, et al. Low density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci U S A* 1989;86:1372-6.
- [59] Toshima S, Hasegawa A, Kurabayashi M, Itabe H, Takano T, Sugano J, et al. Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. *Arteriosclerosis, thrombosis, and vascular biology* 2000;20:2243-7.
- [60] Meisinger C, Baumert J, Khuseynova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation* 2005;112:651-7.
- [61] Araujo JA, Romano EL, Brito BE, Parthe V, Romano M, Bracho M, et al. Iron overload augments the development of atherosclerotic lesions in rabbits. *Arteriosclerosis, thrombosis, and vascular biology* 1995;15:1172-80.

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