

Research Article

Heme Consumption Reduces Hepatic Triglyceride and Fatty Acid Accumulation in a Rat Model of NAFLD Fed Westernized Diet

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Studies have identified that serum-free hemoglobin subunits correlate positively with the severity of nonalcoholic fatty liver disease (NAFLD). However, the role of hemoglobin in the development of NAFLD remains unclear. In the present study, a rat model of NAFLD was developed, using a westernized diet high in saturated fat and refined sugar. Since a “westernized” diet is also high in red meat, we tested the effect of hemoglobin as a dietary source of heme in our model. Sprague-Dawley rats were fed *ad libitum* for 4 weeks either control diet (7% fat), westernized diet (WD, 18% fat + 1% cholesterol), hemoglobin diet (7% fat + 2.5% Hb), or westernized and hemoglobin diet (18% fat + 1% cholesterol + 2.5% Hb). Rats fed WD developed features of NAFLD, including insulin resistance and accumulation of liver fatty acids in the form of triglycerides, increased lipid peroxidation (F₂-Isoprostanes), and liver fibrotic marker (hydroxyproline). Hemoglobin consumption significantly influenced several biomarkers of NAFLD and hepatic biochemistry, suggesting a possible interaction with diet and/or liver lipid pathways. The complex mechanisms of interaction between WD and hemoglobin in our rat model warrants further studies to examine the role of dietary heme on NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as the most common cause of chronic liver disease in many industrialized and developing countries. It is estimated to affect approximately 30% and 10–20% of adults and children, respectively, in the USA and its prevalence is increasing in many countries where consumption of “western diets,” high in fat, is rising [1]. Recently, a prospective epidemiological study reported a significant incidence of NAFLD in nonobese and nonaffluent populations in India [2]. Interestingly, approximately 30% of NAFLD patients have hyperferritinemia [3] and serum hemoglobin subunits α and β increase with severity of the disease in NAFLD patients [4–6].

The prevailing concept of NAFLD is the “two hit” theory that centers on the initial accumulation of fatty acids (FAs) in the liver followed by a sequence of events associated with oxidative stress, lipid peroxidation, and inflammation, resulting in liver injury [7, 8]. However, the exact pathophysiological mechanisms are not fully understood. Therefore, reliable animal models of NAFLD are important to identify the relevant pathophysiology involved in the development of NAFLD in order to develop effective therapeutic strategy.

Many groups have developed and studied experimental models for NAFLD and nonalcoholic steatohepatitis (NASH) using a variety of different high fat diets [9–11]. Evidence indicates that not only the quantity, but also the types of fat and the duration of feeding are important factors in the development of an appropriate model of NAFLD [12–14].

TABLE 1: (a) Composition of diets¹. (b) Fatty acid composition of CD and WD animal diets (means \pm SD) for $n = 5$ animal pellet samples. Fatty acid levels in heme supplemented diets (HbD and W + HbD) were not statistically different to their non-heme comparable diet. $P < 0.001$ for all fatty acids in WD versus CD.

(a)

Diet	CD	WD	HbD	W + HbD
Product NO.	SF07-034	SF07-079	SF07-078	SF07-080
Total fats (%)	7	18 + 1	7	18 + 1
Source	Canola oil	Ghee + cholesterol	Canola oil	Ghee + cholesterol
Hb (%)	—	—	2.5	2.5
Total Fe (mg/Kg)	70	59	144	144
Energy from lipids (%)	15.8	35.0	15.8	35.0
Protein (%)	19.4	19.4	19.4	19.4
Starch (%)	55.0	18.9	55.0	18.9
Sucrose (%)	10.0	34.0	10.0	34.0
Crude fiber (%)	4.7	4.7	4.7	4.7
Digestible energy (MJ/Kg)	16.5	19.0	16.6	19.0

¹Detailed information on these animal diets is available from Specialty Feeds Pty Ltd (<http://specialtyfeeds.com/data/sf07-034.html>).

(b)

Diet	CD	WD
FA (ng/mg diet)		
Caprylic acid (8:0)	27.62 \pm 2.8	187.96 \pm 20.3
Capric acid (10:0)	58.44 \pm 4.7	661.27 \pm 42.7
Lauric acid (12:0)	568.77 \pm 190.6	825.26 \pm 95.2
Myristic acid (14:0)	184.10 \pm 19.1	2301.15 \pm 100.7
Palmitic acid (16:0)	1353.18 \pm 484.4	5392.81 \pm 319.2
Palmitoleic acid (16:1)	18.76 \pm 4.2	144.87 \pm 9.6
Margaric acid (17:0)	14.87 \pm 5.4	94.48 \pm 8.0
Stearic acid (18:0)	868.01 \pm 322.9	2019.55 \pm 205.4
Oleic acid (18:1)	2943.43 \pm 1020.2	1930.05 \pm 148.8
Linoleic acid (18:2)	363.65 \pm 108.9	80.67 \pm 4.4
Linolenic acid (18:3)	329.74 \pm 97.7	55.28 \pm 3.3

This study aims to firstly examine the effect of a westernized diet containing cholesterol, ghee, and refined sugar on the development of NAFLD pathophysiology in a rat model. Ghee is rich in saturated fats (SFs) and cholesterol with a similar fatty acid profile to typical human high fat diets [15]. “Westernized diets” are generally high in red meats, a major source of cholesterol and saturated fat which may contribute to the increased risk of diabetes and cardiovascular disease [16]. Red meat is also a rich source of iron and heme, potential prooxidants and catalysts of peroxidative damage under certain circumstances [17, 18]. However, some *in vitro* studies on hemoglobin have reported protective properties against oxidative injury [19–21] and hemoglobin has not been previously investigated *in vivo* in the context of NAFLD. We therefore studied the role of hemoglobin in the development of NAFLD.

2. Material and Methods

2.1. Materials. All reagents used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Hemoglobin

(Ferrohemoglobin) was from MP Biomedicals (NSW, Australia). Myristic acid (1,2,3-¹³C₃) from Cambridge Isotope Laboratories (Andover, MA). Arachidonic acid and F₂-isoprostanes were from Cayman Chemical (Ann Arbor, MI).

2.2. Animals and Diets. Male Sprague-Dawley rats, 5 weeks old (150 g, $n = 20$), were purchased from Comparative Medicine Centre for Animal Resources (CM-CARE), Singapore. Rats were housed randomly 2 per cage and were allowed water *ad libitum* in an animal facility with a 12 h light-dark cycle. Groups were fed either (1) control diet (CD, SF07-034), a semipure diet formulation based on AIN-93G with 7% fat content from canola oil; (2) westernized diet (WD, SF07-079) where canola oil was replaced with 18% ghee and 1% cholesterol; (3) high hemoglobin diet (HbD, SF07-078) supplemented with 2.5% hemoglobin; and (4) westernized and hemoglobin diet (W + HbD, SF07-080) with 18% ghee, 1% cholesterol and 2.5% hemoglobin (Table 1(a), Glen Forest Specialty Feed, WA). The protocol was approved by the National University of Singapore Institutional Animal Care and Use Committee (IACUC). Animals were euthanized

and excised livers were weighed, washed with phosphate-buffered saline (PBS, 4°C) and snap-frozen in liquid nitrogen and stored at -80°C. Plasma was prepared from collected venous blood by centrifugation and then stored at -80°C with butylated hydroxytoluene (BHT, 20 µM). Livers were scored for their fatty, pale appearance. (1 = normal;) 5 = complete pale, white complexion.

2.3. Plasma Insulin and Alanine Transaminase (ALT) Measurement. Plasma insulin levels and ALT activity were determined using a rat insulin ELISA kit (cat no. EZRMI-13K, Millipore, Billerica, MA) and alanine transaminase activity kit (cat no. 700260, Cayman Chemical, Ann Arbor, MI), respectively, according to the manufacturer's protocol.

2.4. Direct Determination of Liver Triglycerides. Liver triglycerides were determined as described in [22].

2.5. Hydroxyproline Content in Rat Livers. Hepatic hydroxyproline (HP) was measured as an index of collagen content in liver using a method described by Woessner [23]. Briefly, 100 µL of liver homogenate was hydrolyzed in an airtight glass tube by HCl (final concentration, 6 M) for 3 h at 130°C. pH of hydrolyzed sample was adjusted to pH 6.0-7.0 with NaOH (10 M). Hydroxyproline oxidation was initiated by adding chloramine T solution (0.05 M) and incubation for 20 min at 22°C. Excess chloramine T was removed by adding perchloric acid (3.15 M) for 5 min. Finally, *p*-dimethylaminobenzaldehyde solution (20%) was added, vortexed and incubated for 20 min at 60°C. Absorbance was determined spectrophotometrically at 557 nm and hydroxyproline content of liver samples were determined directly from a freshly prepared hydroxyproline standard curve.

2.6. Lipid Extraction and Alkaline Saponification for FA Analysis. 100 µL of liver homogenates (1 mg/mL) or 2 mg of crushed animal chow was weighed into glass tubes and hydrolyzed with concentrated HCl (8 M, 0.5 mL) for 1 h at 80°C. 200 µL of pyrogallol (50 mg/mL) was added to minimize oxidation. Liver homogenates were adjusted to pH 5-6 after hydrolysis using NaOH (10 M) in the presence of K₂HPO₄ buffer (1 M, pH9). Lipid was extracted with 5 mL of ice-cold Folch extraction solvent (chloroform: methanol 2:1, 0.005% BHT). The samples were centrifuged at 3000 rpm for 2 min and 0.5 mL of extraction solvent was carefully transferred into a glass vial. 10 µL (200 ng) of heavy labelled myristic acid was added and samples were dried under N₂ gas. Samples were resuspended in 0.5 mL of PBS and pH was adjusted to 13 with KOH (1 M) and saponified at 20°C with shaking at 75 rpm for 15 h in the dark. The pH was adjusted to 5 with HCl (5 M) in the presence of 0.5 mL of acetate acid buffer (0.4 M, pH4) followed by Folch solvent (1 mL) for 15 min at 20°C. The organic phase was extracted and dried under a stream of N₂ gas and derivatized at 50°C for 1 h with 50 µL of 1:1 BSTFA + 1% TMCS and acetonitrile.

2.7. Gas Chromatography-Mass Spectrometry (GC-MS) FA Analysis. FAs were analyzed using a Hewlett-Packard 5973

mass selective detector interfaced with a Hewlett-Packard 5890II gas chromatograph. Separations were carried out on a fused silica capillary column (30 m × 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (0.33 µm) (Ultra2, Agilent, J & W Scientific). The quadrupole and ion source of the MS were maintained at 150 and 230°C, respectively. Derivatized samples (1 µL) were injected with a 10:1 split into the GC injection port (280°C). Column temperature was increased from 80 to 230°C at 47.5°C/min after 1 min at 80°C and then increased to 235°C at 1°C/min and held for 2 min. The temperature was further increased to 280°C at 50°C/min and held for 0.5 min and then raised to 300°C at 50°C/min and held for 1.5 min. The carrier gas was helium with a flow rate of 0.8 mL/min (average velocity = 55 cm/s). Selected-ion monitoring was performed using the electron ionization mode at 70 eV to monitor one target ion and two or more qualifier ions selected from each compound's mass spectrum to optimize sensitivity and specificity. Relative molar response factors for each analyte were calculated using calibration curves constructed from six different concentrations in triplicate of FAs and showed good linearity ($r^2 > 0.95$).

2.8. GC-MS Quantification of Total Arachidonic Acid and Total Cholesterol. Lipid extractions for the analysis of arachidonic acid, cholesterol, and F₂-isoprostanes of liver samples using GC-MS were performed as described by Jenner et al. [24].

2.9. Statistical Analysis. Differences between means were evaluated using a one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for multigroup analysis (* $P < 0.05$ versus CD, ** $P < 0.01$ versus CD, *** $P < 0.001$ versus CD, # $P < 0.05$ versus WD, ## $P < 0.01$ versus WD, \$\$\$ $P < 0.001$ versus HbD, \$\$\$ $P < 0.001$ versus HbD considered as significant).

3. Results

3.1. Body and Liver Weights. Rats fed a westernized diet (WD) for 4 weeks showed a trend of higher body and liver weights, but this was not statistically significant compared to control diet (CD, Table 2). Growth rate of the rats in each group did not differ significantly from each other (data not shown). Livers from rats fed diets enriched in fat appeared white and fatty (scale 5) compared to normal livers (scale 1) of CD and hemoglobin diet (HbD) fed rats. The presence of heme in WD reduced the fatty appearance of rat livers (scale 3).

3.2. FA Profile of Animal Diets. As expected, WD supplemented with ghee contained increased levels of saturated fatty acids (SFAs) such as palmitic, myristic, stearic, and capric acids. In the CD and HbD comprising canola oil, we found more oleic, linoleic and linolenic acids (Table 1(b)). Using the GC-MS for FA analysis, the recovery of total FAs in animal chow and liver tissue was calculated to be 80.2-86.0%.

3.3. WD Increases Hepatic FA and Triglyceride Levels. GC-MS analysis confirmed the accumulation of FAs in rat livers as

TABLE 2: Weights of animals and livers, plasma ALT activity, liver triglyceride, cholesterol and F₂-isoprostanes levels and appearance of liver after 4 weeks of feeding¹.

Diet	CD	WD	HbD	W + HbD
Body wt (g)	431 ± 28	469 ± 26	407 ± 59	487 ± 72
Liver wt (% body wt)	4.3 ± 0.5	4.8 ± 0.4	4.0 ± 0.4	4.5 ± 0.5
Plasma ALT activity (U/mL, fold change)	1.0 ± 0.3	1.69 ± 0.9	0.65 ± 0.2	0.64 ± 0.3 [#]
Liver triglycerides (mg/g liver)	5.0 ± 0.4	21.0 ± 2.0 ^{***}	5.2 ± 0.5	17.8 ± 1.9 ^{***\$}
Liver cholesterol (mg/g liver)	2.05 ± 0.3	2.33 ± 0.3	1.77 ± 0.4	2.02 ± 0.3
Liver F ₂ -isoprostanes (pg/μg AA)	7.5 ± 1.2	15.7 ± 2.5 ^{***}	9.6 ± 2.5	13.7 ± 2.2 ^{**}
Appearance of liver ²	1	5	1	3

¹Data are expressed as means ± SD for $n = 5$ rats. ²For appearance of liver, 1: healthy normal, 5: fatty and white. ^{*} $P < 0.01$, ^{***} $P < 0.001$ versus CD, ^{\$\$\$} $P < 0.001$ versus HbD, [#] $P < 0.05$ versus WD. ALT; alanine transaminase.

TABLE 3: FA profile of rat livers¹.

Diet	CD	WD	HbD	W + HbD	P
FA (ng/mg protein)					
Caprylic acid (8:0)	137 ± 21	201 ± 20 [*]	155 ± 34	138 ± 18 [#]	<0.05
Capric acid (10:0)	169 ± 30	414 ± 35 ^{**}	195 ± 41	225 ± 48 [#]	<0.01
Lauric acid (12:0)	1837 ± 330	2715 ± 457 ^{**}	1906 ± 266	2199 ± 374	<0.01
Myristic acid (14:0)	959 ± 395	3379 ± 567 ^{**}	875 ± 387	1697 ± 285 [#]	<0.01
Palmitic acid (16:0)	11799 ± 2943	37621 ± 10131 ^{**}	9166 ± 1447	17181 ± 3319 [#]	<0.01
Palmitoleic acid (16:1)	856 ± 400	7419 ± 2863 ^{**}	426 ± 165	2726 ± 565 [#]	<0.01
Margaric acid (17:0)	110 ± 34	436 ± 47 ^{**}	102 ± 27	252 ± 24 [#]	<0.01
Stearic acid (18:0)	8741 ± 623	10360 ± 1079	8327 ± 917 [#]	10031 ± 812	<0.05
Oleic acid (18:1)	6881 ± 3257	30407 ± 9897 ^{**}	4513 ± 1210	12578 ± 4198 [#]	<0.01
Linoleic acid (18:2)	894 ± 252	1543 ± 361	678 ± 52 [#]	961 ± 153	<0.01
Linolenic acid (18:3)	207 ± 74	212 ± 54	170 ± 87	120 ± 41	=0.087
Docosahexaenoic acid (22:6)	1866 ± 220	3557 ± 553 ^{**}	1358 ± 448	2066 ± 402 [#]	<0.01

¹Data are expressed as means ± SD for $n = 5$ rat livers. ^{*} $P < 0.05$ versus CD, ^{**} $P < 0.01$ versus CD [#] $P < 0.05$ versus WD, ^{##} $P < 0.01$ versus WD are significantly different.

shown in Table 3. Significant differences ($P < 0.01$ and $P < 0.05$) were observed in livers of WD-fed rats compared to CD in all FAs measured except stearic acid (18:0), linoleic acid (18:2) and linolenic acid (18:3). HbD did not significantly alter the FA profile compared to CD. However, the presence of Hb with WD significantly reduced hepatic accumulation of FAs compared to WD with the exception of lauric, stearic, linoleic, and linolenic acids. Liver triglycerides (TGs) increased in rats fed WD and W + HbD compared to CD (Table 2, $P < 0.001$), whilst WD with added heme reduced liver TGs compared to WD alone ($P < 0.05$).

3.4. WD Increases Liver F₂-Isoprostanes, Plasma Insulin Level and Alanine Transaminase (ALT) Activity. Liver F₂-isoprostanes and plasma insulin concentrations of the rats fed the WD were significantly higher than those of the rats fed CD (Table 2, Figure 1(a)). W + HbD showed a trend of decreasing liver F₂-isoprostanes and plasma insulin levels compared to WD, but neither were statistically significant. W + HbD significantly reduced plasma ALT activity compared to WD alone, although the increase of plasma ALT activity by WD alone was statistically not significant compared to CD (Table 2).

3.5. Liver Total Iron, Heme, and Hydroxyproline Content. Compared to CD, WD and HbD did not affect liver total iron content whilst W + HbD rat livers were significantly higher compared to HbD (Figure 1(b)). In contrast, liver heme content increased with HbD compared to CD, whilst other diets were not significantly different (Figure 1(c)). There was a significant increase in liver collagen levels as determined by hydroxyproline contents in rats fed with WD compared to CD (Figure 1(c)). However, liver HP level was reduced significantly in W + HbD compared to WD.

4. Discussion

Several experimental animal models have been developed to study the mechanisms responsible for the onset of NAFLD and NASH [25]. Diets rich in polyunsaturated and/or monounsaturated fatty acids are generally accepted to offer a beneficial effect on insulin sensitivity as compared to SFAs [26, 27]. However, several animal studies demonstrate that excess intake of “good” unsaturated fats can actually be detrimental, highlighting the importance of the amount and duration of fat feeding in the development of a NAFLD model [9–11].

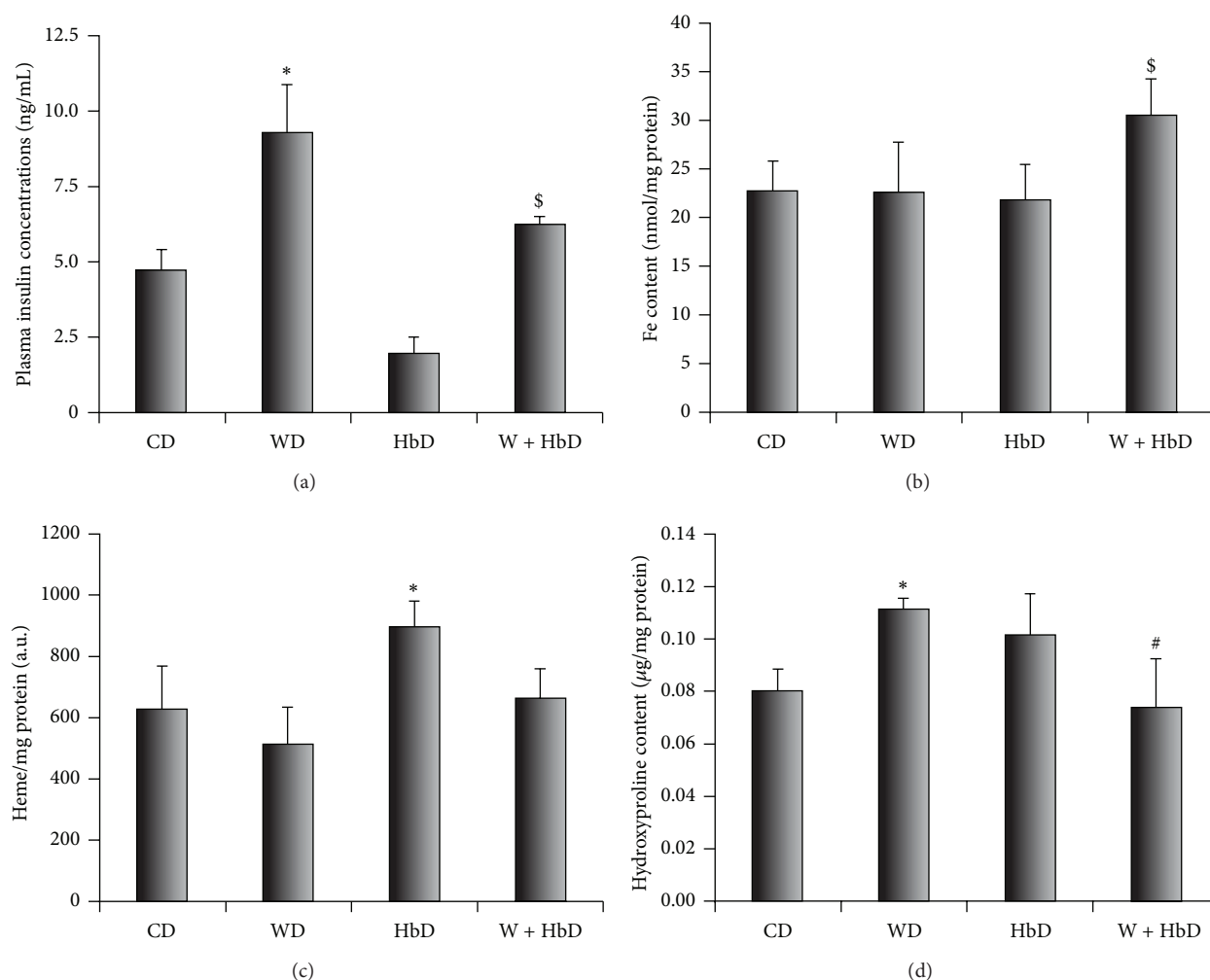


FIGURE 1: Plasma insulin, hepatic total iron, heme and hydroxyproline contents in rats. Rats were fed CD, WD, HbD and W + HbD for 4 weeks *ad libitum*. (a) WD increases plasma insulin levels in rats compared to CD. This effect was attenuated in W + HbD fed rats. (b) Total iron content was elevated in liver of animals on W + HbD compared to HbD. (c) Liver heme and (d) hydroxyproline content were increased in animals on HbD and WD, respectively compared to CD. Data are means \pm SD for $n = 4-5$ samples. CD: control diet, WD: westernized diet, HbD: hemoglobin diet, W + HbD: westernized and hemoglobin diet. * $P < 0.05$ versus CD, # $P < 0.05$ versus WD, \$ $P < 0.05$ versus HbD. a.u.: arbitrary unit.

A typical “westernized diet” is rich in SFAs and cholesterol and provides about 35–40% energy from fat [26]. GC-MS analysis of FA composition in our WD confirmed that using ghee as an alternative to plant oil provided a more physiologically relevant “westernized diet” in SFA content and fat derived energy. Detailed GC-MS FA profiling of diets is highly beneficial and often not performed or reported in previous NAFLD animal studies. Further GC-MS analysis ensured that WD quality was maintained throughout the 4 week study and that our storage conditions (4°C under vacuum) prevented FA oxidation. Lipid is susceptible to oxidation under poor storage environments and the presence of lipid peroxidation products is highly undesirable due to their complex biological and pathophysiological activities, which can confound the interpretation of these feeding studies [28].

Initial development of NAFLD is believed to involve hepatic lipid accumulation, commonly referred to as the first hit in the “two hit” hypothesis of NAFLD [7]. Feeding overweight nondiabetic subjects a high fat diet for 10 days has been shown to increase liver fat content by 35% [29]. In patients with NAFLD, dietary fat intake accounted for about 15% of intrahepatic lipid accumulation [30]. In our study, feeding rats with a WD produced fatty livers and significantly increased levels of liver FAs, mostly stored as TGs, satisfying a fundamental criterion on NAFLD. Insulin resistance in WD rats of our study, indicated by significantly raised plasma insulin, is likely to be the consequence of hepatic FA accumulation [31–33]. Interestingly, WD did not result in significantly elevated hepatic cholesterol levels, which may be due to different regulation of separate metabolic pathways that control hepatic uptake, synthesis, storage and export of

cholesterol compared to FAs. It is well documented that rats fed high cholesterol diets are less prone to hypercholesterolemia than other animal models [24, 34–36].

The second hit in NAFLD involves multiple effects arising from oxidative stress and lipid peroxidation. Lett eron and colleagues have shown that hepatocytes with accumulated fat are susceptible to lipid peroxidation [37]. Oxidized cholesterol has been shown to contribute to the development of atherosclerosis in ApoE and Ldl receptor-deficient mice [38]. Accumulation of F₂-isoprostanes and collagen content (measured as hydroxyproline content) provides further evidence of oxidative stress in this model.

Recently, several groups have reported that serum levels of free hemoglobin α and free hemoglobin β reflect the severity of NAFLD and thus provide an additional biomarker for the disease [4–6]. Moreover, significant changes in iron metabolism have been associated with NAFLD. Approximately 30% of NAFLD patients have hyperferritinemia [3] and; interestingly, iron depletion by phlebotomy treatment reduced features of NAFLD [39]. Conversely, impairment of iron sensing or regulatory proteins has an opposite effect on iron depletion in NAFLD patients [40]. The effect of dietary hemoglobin was complex but important differences were observed in W + HbD compared to WD. Intriguingly, our data in WD rats suggest a possible “protective” effect of dietary hemoglobin on livers by decreasing FA and TG accumulation, collagen content, plasma ALT activity and insulin levels. The mechanism of these effects is unclear, but suggests that dietary hemoglobin does not elicit a direct “prooxidant” activity in the liver. Indeed, liver F₂-isoprostanes (the gold standard biomarker of lipid peroxidation) were unaffected by addition of heme to our diets. Liu et al. reported increased expression of hemoglobin subunits in HepG2 and HEK293 cells upon hydrogen peroxide challenge and hemoglobin overexpression suppressed oxidative stress in HepG2 cells [19]. Others have also showed that hemoglobin has antioxidative functions under different conditions [20, 21]. Our data suggests that dietary fat may influence processes that control hepatic levels of heme and iron, in particular elevating iron at the expense of heme. Moreover, rabbits on a high cholesterol diet displayed microvascular damage associated with iron accumulation and lipid peroxidation in the liver [36]. The possibility that the quantity and type of dietary fat have different effects on the uptake, metabolism and transport of heme and/or iron, merits more detailed investigations to examine potential associated mechanisms.

In conclusion, a relevant NAFLD animal model was established through feeding rats a diet containing increased levels of saturated fat and refined sugar. This animal model displayed evidence of increased fat accumulation in liver, insulin resistance, and mild oxidative stress with elevated F₂-isoprostanes and collagen deposition. Another advantage of the current study was that rats retained their voluntary oral feeding patterns with the WD. Supplementation of hemoglobin in WD indicates that dietary heme does not promote NAFLD or liver oxidative stress. We believe that this animal model provides a useful tool to elucidate the mechanisms underlying the development of NAFLD.

Abbreviations

ALT:	Alanine transaminase
AA:	Arachidonic acid
CD:	Control diet
FAs:	Fatty acids
HbD:	Hemoglobin diet
WD:	Westernized diet
W + HbD:	Westernized and hemoglobin diet
HP:	Hydroxyproline
TGs:	Triglycerides.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Soon Yew Tang, Irwin Kee M. Cheah, Pei Ern Ng, Aina Hoi, and Andrew M. Jenner contributed equally to this work.

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