Iron deficiency (ID) is widespread all over the world, and the prevalence of anemia is approximately 33% in 2010 [1] although iron is an essential trace element for humans. The prevalence of marginal ID (MID) is higher than that of ID [2]. There is a high incidence of ID in obese individuals [3] as well as frequent ID in diet-induced obese animals [4–7], suggesting that ID participates in some aspects of metabolic syndrome, especially nonalcoholic fatty liver disease (NAFLD). Excessive energy consumption would enhance lipid accumulation [8] and hepatic steatosis is found in most of the cases [9]. Substantial lipid accumulation in the liver is a hallmark of NAFLD, which leads to subsequent development of cirrhosis, liver cancer, and increased mortality [10]. The causal relationship between ID and hepatic steatosis needs to be clarified.

As the liver is the major organ for iron storage and lipid metabolism, there might be a considerable involvement of iron in the regulation of lipid metabolism in the liver. Actually, some animal studies revealed that ID is associated with liver lipid accumulation. For example, ID induces liver triglyceride accumulation [11] and upregulation of lipogenic genes in the liver [12]. However, such ID often induces severe clinical symptoms with anorexia and growth retardation [13]. In an epidemiological study, the incidence of ID without anemia, namely, MID, was nearly four times higher than that with anemia [2]. It remains unknown whether MID also influences liver lipid accumulation.

In the literature, ID without anemia impairs cognitive function [14] and endurance capacity [15,16] in clinical trials, as well as heart function [17] in a culture experiment. These observations suggest that MID could possibly modulate cellular functions. Since the liver is responsible for iron metabolism, a marginal reduction in iron concentration might influence lipid metabolism in the liver. The aim of the present study was to establish diet-induced MID in rats and to investigate whether MID influences liver lipid accumulation in combination with dietary sucrose.

Material & methods
Animal experiments
The study was approved by the Institutional Animal Care and Use Committee of National Corporation Hokkaido University (approval number: 14–0026 and 17–0119), and all animals were maintained in accordance with the Hokkaido University Manual for Implementing Animal Experimentation. Male Wistar rats (3 weeks old; Japan SLC Inc., Hamamatsu, Japan) were housed individually in a controlled environment at 22 ± 2°C and 55 ± 5% humidity. The light period was from 08:00 to 20:00. The rats had free access to food and water for the entire study period. In the first experiment, the rats were acclimated on an AIN-93G-based iron adequate...
Diet composition in the second experiment.

<table>
<thead>
<tr>
<th></th>
<th>IA</th>
<th>MID</th>
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<tbody>
<tr>
<td>Casein*</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Dextrin*</td>
<td>529.5</td>
<td>529.5</td>
<td>529.5</td>
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<tr>
<td>Sucrose*</td>
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<tr>
<td>Soybean oil*</td>
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<tr>
<td>Cellulose*</td>
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<tr>
<td>Mineral mixture for ID*</td>
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<tr>
<td>Mineral mixture for MId*</td>
<td>-</td>
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</tr>
<tr>
<td>Vitamin mixture*</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>L-Cystine*</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline hydrogen tartrate*</td>
<td>2.5</td>
<td>2.5</td>
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</tr>
</tbody>
</table>

\*NZMP Acid Casein (Fonterra Co-Operative Group Limited, Auckland, New Zealand), \*TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan), \*Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan, \*J-Oil Mills, Inc., Tokyo, Japan, \*Crystalline cellulose (Celulos PH-102, Asahi Kasei Chemicals Corp., Tokyo, Japan), \*AIN-93G Mineral mixture [29].

The mineral mixtures for IF (iron-free), and the MID diet (IA:ID = 1:1, w/w). In the second experiment, male Wistar rats (3 weeks old) were acclimated on the IA diet for 1 week and then divided into four groups. Each group was fed one of four diets for 3 weeks as follows: the IA diet, the MID diet, a high sucrose-based IA diet (HS-IA) and a HS-based MID diet (HS-MID) (Table 2). In each experiment, body weight and food intake were measured every day. The aortic blood plasma was collected under anesthesia with sodium pentobarbital (50 mg/kg body weight) into a syringe containing heparin (final concentration at 50 IU/mL) and aprotenin (final concentration at 500 KIU/mL). Plasma was separated by centrifugation at 2,000 × g for 10 min at 4°C. After the rats were euthanized by exsanguination, the liver and epididymal adipose tissue were collected and weighed. The collected plasma and tissues were stored at −80°C until analysis. All rats were euthanized by exsanguination within three hours (start from 10 AM). Food deprivation was not performed in all experiments.

Biochemical analysis

For lipid extraction, 100 mg of liver was immersed in an extraction solution (chloroform:methanol = 2:1 [19]) for 2 days. The extracts were collected and evaporated in a fume hood. Lipids in the extract were dissolved in 2-propanol for measurement. The activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by using a transaminase CII-test Wako kit (Wako Pure Chemical Industries, Osaka, Japan). Cholesterol and triglyceride levels were measured by using a cholesterol E-test Wako kit (Wako) and triglyceride E-test Wako kit (Wako), respectively. The plasma concentrations of iron, ferritin, glucose and insulin were determined by using an Iron Assay kit LS – Ferrozine method (Metallogenics, Chiba, Japan), a Ferritin (Cat) ELISA Kit (Abnova, Taipei, Taiwan), a Glucose CII-Test Wako (wako) and a LBIS Insulin-Rat-T (Shibayagi, Gunma, Japan), respectively. Blood hemoglobin level was determined by using a Hemoglobin Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA).

Measurement of liver glucose

Liver tissue (20 mg) were homogenized and sonicated with 500 µL of cold PBS on ice. After centrifugation at 14,000 × g for 10 min, supernatants were collected and properly diluted to measure glucose level by the kit described above. The values were normalized by protein level in the supernatants determined using by a Takara BCA Protein Assay Kit (Takara Bio, Shiga, Japan).

Liver iron analysis

Liver iron was extracted as previously described [20] with minor modifications. Briefly, the freeze-dried livers were weighed and milled to fine powder. The liver powder was dry-ashed at 550°C for 3 h in a polarized muffle furnace (TMF-3200; Tokyo Rikakikai, Tokyo, Japan) and heated with 20% nitric acid until evaporated, and a 3% nitric acid solution was added to the ash. The iron concentration in the extracted samples was measured with an atomic absorption spectrophotometer (Z-5310; Hitachi High-Technologies Corporation, Tokyo, Japan).
Liver mRNA expression was measured using RT-qPCR. Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, and the RNA concentration was measured with a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA). The complementary DNA was synthesized from 1 µg of the RNA using ReverTraAce® qPCR RT master mix with gDNA remover (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer’s instructions. The qPCR was performed using an Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with TaqMan Gene Expression Assays (Rn03302271_gH for ribosomal protein lateral stalk subunit P0 (Rplp0), Rn01495769_m1 for sterol regulatory element binding protein-1c (Srebp1c), Rn01463550_m1 for fatty acid synthase (Fas), Rn00580702_m1 for carnitine palmitoyltransferase 1 (Cpt1), and Rn00580432_m1 for interleukin-1 beta (Il1β) (Life Technologies, Carlsbad, CA, USA). Relative expression levels were calculated for each sample after normalization to those of Rplp0 as a reference gene using the standard curve method.

DNA extraction and analysis of mitochondrial DNA (mtDNA)

Liver DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). The amount of mtDNA and nuclear DNA (nDNA) was determined by real-time qPCR [21] with specific primer pairs for mitochondrial gene encoded 16S RNA (Mt-Rnr2, forward: 5’-ACTCGTTAGGCCCCAACAGGG-3’, reverse: 5’-CCGTTAACTTTAGTCACTG-3’, annealing temperature at 62°C, 152 bp) and nuclear gene encoded Rplp0 (forward: 5’-AGGGCCTTAGCGAAGAGACC-3’, reverse: 5’-GACAGCTCTTAAACAGGAGGTA-3’, annealing temperature at 62°C, 236 bp). To determine the number of cells, we calculated the ratio of the mtDNA against the nDNA as an index for the number of mitochondria per cell. For PCR, 2 µL of sample DNA (5 ng/µL) was mixed with 0.25 µL of each primer (10 µM), 4.5 µL of nuclease-free water, 0.25 µL of ROX Reference Dye II marker, and 6.25 µL of SYBR Premix Ex Taq. The reaction was started at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 61°C for 15 s, and extension at 72°C for 10 s. Amplification curves were analyzed using an Mx3000P real-time PCR system (Stratagene) to determine the mtDNA/nDNA ratio in each sample.

Statistical analysis

All data are presented as the mean ± SEM. In the first experiment, differences in all parameters compared with the IA group were determined using Dunnett’s test. In the second experiment, two-way ANOVA (iron and sucrose) was used to evaluate differences in all parameters. When the test gave an interaction (iron × sucrose), a post hoc test (Student’s t-test) was applied. The statistical analysis was performed using JMP version 12.0 (SAS Institute Inc., Cary, NC, USA).

Results

Comparable growth and food intake in the rats fed MID and IA diets

To confirm whether the MID diet induces ID without anemia, we fed the rats with the experimental diet for three weeks in the first experiment. A significant reduction in food intake in ID-fed rats was detected from day 3 compared to that in the IA-fed rats (Figure 1(a)), and the cumulative food intake also decreased significantly in the ID-fed rats (284 ± 4 g) compared with that in the IA-fed rats (326 ± 7 g). Similarly, a significant difference in body weight was observed in the ID-fed rats compared with that in the IA-fed rats from day 3 (Figure 1(b)), and the final body weight was significantly reduced in the ID-fed rats (213 ± 3 g) compared to that in the IA rats (238 ± 3 g). These results are consistent with...
previous observations in severe ID experiments [13]. Liver weight was significantly decreased in the MID (4.1 ± 0.2 g/100 g body weight) and ID-fed rats (3.9 ± 0.2 g/100 g body weight) compared to that in the IA-fed rats (4.4 ± 0.3 g/100 g body weight). Epididymal adipose tissue weight was significantly reduced in the ID-fed rats (1.7 ± 0.3 g/100g body weight) but not in the MID-fed rats (2.0 ± 0.3 g/100 g body weight) compared to that in the IA-fed rats (2.1 ± 0.3 g/100 g body weight). The cumulative food intake and final body weight of the ID-fed rats were 87.1% and 89.8% of those of the IA-fed rats, respectively. Notably, no difference was found in daily food intake and body weight between the MID- and IA-fed rat groups during the experiment. These results suggest that food consumption and growth in the MID-fed rats were almost comparable with those in the IA-fed rats.

**The MID diet induces ID without anemia**

To evaluate whether the MID diet reduced hepatic iron concentration without anemia, we analyzed iron-related parameters (Figure 2). The ID diet reduced iron concentrations in both plasma and liver, accompanied by decreased hematocrit and hemoglobin levels as well as plasma ferritin concentration. On the other hand, the MID diet decreased plasma ferritin and iron concentrations in the plasma and liver as shown in Figure 2(a-c), but no induction of anemia was observed in the MID-fed rats, judging by the hematocrit and hemoglobin levels (Figure 2(d,e)). Therefore, we determined that the MID diet successfully induces ID in the plasma and liver without anemia. On the other hand, no difference was observed in liver triglyceride concentration between the rats fed MID (13.2 ± 2.5 mg/g liver) and IA diets (12.6 ± 2.1 mg/g liver).

**The MID diet promotes hepatic lipid accumulation in response to the HS diet**

As the consumption of the MID diet was still insufficient to induce hepatic lipid accumulation in the first experiment, we hypothesized that some lipogenic stimuli are required to cause lipid accumulation in MID. It has been reported that an increase in expression of lipogenic genes in response to ID was observed on an AIN-76A diet but not on an AIN-93 diet [22]. We chose a HS diet as a lipogenic-promoting diet [23,24] because the major carbohydrate source in AIN-76A and AIN-93 is sucrose and starch, respectively [18]. As a result, no differences were observed in food intake, body weight, and epididymal adipose tissue weight among all groups (Table 3). The HS diet significantly increased liver weight (two-way ANOVA, P < 0.0001) regardless of the iron concentration in the diet. The MID diet decreased liver iron concentration without a reduction in hemoglobin level (Figure 3(a-c)), although a reduction in plasma iron concentration was found only with the control diet in response to a decrease in dietary iron (Figure 3(b)). These results suggest that the MID diet can also induce hepatic ID in the HS diet. Intriguingly, the MID diet significantly increased liver triglyceride concentration only in the HS-fed condition (Figure 4(a)). Two-way ANOVA analysis revealed a significant interaction between sucrose and iron in the values of liver triglycerides. Although the HS diet significantly increased plasma TG, there was no difference in the other parameters related to lipid metabolism (Figure 4(b-d)). We determined plasma transaminase activities (Figure 4(e-f)) as lipotoxicity markers associated with liver injury [25]. Although HS significantly elevated plasma ALT, the alteration was not necessarily associated with liver triglyceride concentration (Figure 4(f)). No difference was observed in AST activity (Figure 4(g)) among the groups.

**The MID diet augments fas gene expression in response to the HS diet**

Severe ID is associated with an increase in the hepatic expression of lipogenic genes [12]. Thus, we measured expression levels of the genes related to lipid synthesis as well as inflammation (Figure 5). Notably, consistent with liver lipid accumulation, a significant increase was found in Fas expression in the MID-fed rats in response to HS (Figure 5(a)). In contrast, no change was observed in the expression of Srebp1c and Cpt1 (Figure 5(b-c)). The HS significantly increased aortic plasma glucose but not MID (Control: 6.9 ± 0.6, MID: 7.2 ± 0.7, HS:

![Figure 2](https://academic.oup.com/bbb/article/82/12/2140/5955803)
9.1 ± 1.7, HS–MID: 9.3 ± 1.6, two-way ANOVA, P = 0.0005 for HS). No difference was observed in liver glucose and aortic insulin level among the groups (data not shown). We also measured interleukin-1β (Il1β) gene expression as a marker of inflammation, but no difference was observed (Figure 5(d)). We measured the relative amount of mtDNA as liver mitochondria are the major organelle for fatty acid β-oxidation. As a result, the mtDNA/nDNA ratio tended to decrease in response to a reduction in dietary iron concentration (Figure 5(e), P = 0.0807).

### Discussion

Reduction in hepatic iron concentration is expected to influence liver function in ID, even in the absence of anemia. Previous studies have shown that hepatic lipid accumulation was accompanied by anemia in the ID model [11]. However, the incidence of MID is more widespread than severe ID [2]. The primary aim of this study was to establish a reasonable MID model. The present study demonstrated that the diet containing a 50% concentration of the iron content in

#### Table 3. Growth parameters and tissue weight in rats of the second experiment.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HS</th>
<th>Two-way ANOVA, P value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IA</td>
<td>MID</td>
<td>Iron (I)</td>
</tr>
<tr>
<td>Growth parameters (g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Initial body weight</td>
<td>121 ± 2</td>
<td>121 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Total food intake</td>
<td>287 ± 13</td>
<td>310 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Final body weight</td>
<td>228 ± 7</td>
<td>232 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Tissue weight (g 100g body weight)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.50 ± 0.1</td>
<td>4.30 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Epididymal adipose tissue</td>
<td>1.93 ± 0.1</td>
<td>1.84 ± 0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Means values with their standard errors.

#### Figure 3. Iron-related parameters in the rats fed the MID diet with or without HS for 3 weeks (a) Liver iron, (b) Plasma iron, (c) Hemoglobin in the rats fed control or the HS diet combined with either IA or MID diet for 3 weeks. Data are presented as the mean ± SEM.

#### Figure 4. Liver lipids, plasma lipids, and plasma transaminase activity in the rats fed the HS-MID diet for 3 weeks (a) Liver triglyceride (TG) concentration, (b) Plasma TG concentration, (c) Liver cholesterol (Cho), (d) Plasma Cho concentration, (e) Plasma ALT activity and (f) Plasma AST. White and gray bars represent the data of the rats fed the IA and MID diets, respectively. Values are presented as the mean ± SEM. Asterisk represents significant differences from the data of the IA-fed rats and the HS-fed rats (P < 0.05, n = 6).
the AIN-93 mineral mixture successfully induced a reduction in iron concentration in the liver and plasma without anemia. In contrast, the ID diet induced anemia as confirmed by the reduction in hemoglobin or hematocrit levels, which is in line with the results of previous study [11]. The major influence of the ID diet is retarded growth with reduction in food intake [13]. Importantly, the present study demonstrated no influence of the MID diet on voluntary food intake and growth. In humans, we do not usually notice and are not able to evaluate the precise iron consumption rate in daily life, which might increase the prevalence of ID without anemia. The MID-fed rats are considered to reflect presymptomatic conditions in human ID without anemia.

Previous reports have shown that severe ID alone does not cause hepatic lipid accumulation even with anemia [22,26–28]. Similarly, we found that the reduction in iron concentration in the plasma and liver induced by the MID diet was insufficient to cause hepatic lipid accumulation. Notably, hepatic lipid accumulation or hyperlipidemia in ID was only observed with the ingestion of a sucrose-rich diet [11,12,29–31]. In accordance with these studies, Davis and colleagues [22] demonstrated that ID with anemia increases serum triglycerides and hepatic lipogenic gene expression only in the rats fed with an AIN-76 diet but not with an AIN-93 diet. Sucrose, a major carbohydrate source of the AIN-76 diet [18], is a potent lipogenic carbohydrate containing fructose that enhances hepatic lipid accumulation compared to glucose [32–36]. Thus, we speculated that MID participates in hepatic lipid accumulation in response to the HS diet. Indeed, we confirmed that the MID diet is sufficient to promote hepatic lipid accumulation in response to the HS diet. The incidence of non-obese fatty liver has gradually increased [37]. Similarly in the present study, the liver lipid accumulation in the HS–MID-fed rats was independent of epididymal adipose tissue weight. The liver TG accumulation in the HS–MID-fed rats was calculated to only 3.2% of the whole liver, suggesting mild liver TG accumulation at earlier phase of fatty liver development.

Liver lipid accumulation is considered to be mainly due to an imbalance of de novo lipogenesis and fatty acid catabolism. Because Fas catalyzes the terminal step in the biogenesis of fatty acids, Fas expression is thought to be a marker of lipogenesis. We found that enhancement of Fas expression was closely associated with liver triglyceride accumulation in the HS-MID-fed rats, suggesting that liver ID promoted liver triglyceride accumulation partially via enhancement of Fas gene expression in the presence of sucrose. Interestingly, levels of Srebp1c, a major positive regulator of Fas expression [38,39], failed to increase in each treatment, suggesting that Srebp1c expression was not responsible for the increase in Fas expression in the HS-MID-fed rats. Glucose is the major ligand for another key regulator in the expression of the Fas gene, carbohydrate-responsive element binding protein (Chrebp) [40].
Several reports have suggested that ID animals utilize carbohydrate as an energy source instead of fat [12,41–43]. Additionally, an increase in hepatic glucose production has been reported in ID anemic rats [41]. Hepatic iron depletion by deferoxamine, an intracellular iron chelator, enhances hepatic glucose uptake [44]. However, the MID did not affect liver glucose and aortic insulin levels although the HS significantly increased aortic plasma glucose level, suggesting that liver ID by the MID diet is not sufficient to impair glucose metabolism. These results should be carefully interpreted because we did not perform fasting before collecting the samples. Alternatively, it has been reported that replacement of dietary glucose with fructose further increases Chrebp activity [45]. It is possible that liver ID and fructose synergistically activate Chrebp, resulting in an upregulation of Fas gene expression because of the pivotal difference in dietary fructose level between the control diet and the HS diet.

The increase in lipogenesis seems to be responsible for hepatic lipid accumulation as there is no alteration in Cpt1 gene expression. In contrast, Masini and colleagues [46] reported that liver lipid accumulation is associated with mitochondrial dysfunction in ID rats. Although we did not perform a functional analysis of mitochondria, we found that the number of mitochondria in the liver tended to decrease with the MID diet. The MID diet may impair mitochondrial function and partially participate in the increase in liver lipid accumulation in the rats fed the HS-MID diet.

Free fructose and sucrose appear to have a similar pathological effect [47] and fructose is considered to be more lipogenic than glucose [32,48]. In humans, average fructose intake was estimated at 49 g/day [49], which is equivalent to 196 kcal/day. As the energy intake of human adults is 2,000–2,500 kcal/day, daily energy intake from fructose (about 200 kcal/day) estimated to be at 8–10%. In the present study, the rats fed the HS diet appear to consume quite a high level of fructose (32% of their total energy intake). However, it is common to use diet containing a high dose of fructose (~66%) to develop metabolic syndrome in a short period [50]. In addition, rodents may have resistance against fructose ingestion than humans. Jang et al. [51] demonstrated that small intestine initially metabolizes ingested fructose rather than liver in mice. Indeed, ingestion of fructose (0.25 g/kg, as a 1:1 mixture of glucose and fructose) in mice would not appear in portal blood as fructose [51]. In contrast, in humans, ingestion of a lower dose of fructose (0.1–0.15 g fructose/kg if body weight was 50–75 kg, as a 10:1 mixture of glucose and fructose) increased in circulating fructose concentration nearly two-fold [52]. Hence, rodent liver may be less sensitive to fructose ingestion than humans due to high metabolic capacity of the small intestine as some researchers proposed [51,53]. Furthermore, there are some reports showing that humans consume a high level of dietary fructose as follows. In NAFLD patients, consumption of fructose is more than twofold higher than healthy individuals, amounting to 90 g from sweetened beverages alone [24]. Ventura et al. [54] demonstrated that high fructose corn syrup (HFCS), a major source of fructose in sweetened beverages, contains higher fructose amount than that of disclosures from producers. They assumed actual human fructose intake may be around 18% higher than previously estimated by Marriott and colleagues [49].

In conclusion, we established ID without anemia by ingestion of the MID diet and found an increase in hepatic lipid accumulation in the MID-fed rats in the presence of large amounts of dietary sucrose accompanied by the enhancement of Fas gene expression. Epidemiological studies showed the prevalence of inadequate iron status [55] and an inverse correlation between hepatic iron concentration and hepatic steatosis in NAFLD patients [56]. The present study suggests that liver-specific ID in the presence of enormous fructose triggers hepatic lipid accumulation and that iron is a potent modulator for preventing NAFLD.

**Author Contribution**

S.H. and S.I. designed the experiments. S.H. performed experiments. All authors discussed the data. S.H. and S.I. wrote the paper.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**References**


