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1 **Marginal iron deficiency enhances liver triglyceride accumulation in rats fed a**
2 **high-sucrose diet**

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16

17 **Abstract**

18 We investigated whether marginal iron-deficiency (MID) without anemia influences liver
19 lipid accumulation in rats. Ingestion of a MID diet in which the iron concentration was half of
20 AIN-93 formulation (iron-adequate, IA) for 3 weeks decreased liver iron concentration
21 without anemia. We then evaluated the influence of the MID diet on liver lipid accumulation
22 in combination with a high-sucrose (HS) diet and confirmed that the HS-MID diet
23 successfully decreased liver iron concentration without anemia. Additionally, a significant
24 increase in liver triglyceride concentration was found, accompanied by upregulation of
25 hepatic fatty acid synthase expression in the rats fed the HS-MID diet compared to those in
26 the rats fed an HS-IA diet, although no difference was observed in plasma transaminase
27 activity and hepatic interleukin-1 β expression. These results suggest that MID enhances de
28 novo lipid synthesis via upregulation of lipogenic gene expression in combination with
29 sucrose in the diet.

30 **Keywords: marginal iron deficiency; liver lipid accumulation; fatty acid synthase**

31

32 **Introduction**

33 Iron deficiency (ID) is widespread all over the world, and the prevalence of anemia is
34 approximately 33% in 2010 [1] although iron is an essential trace element for humans. The
35 prevalence of marginal ID (MID) is higher than that of ID [2]. There is a high incidence of ID
36 in obese individuals [3] as well as frequent ID in diet-induced obese animals [4–7],
37 suggesting that ID participates in some aspects of metabolic syndrome, especially
38 nonalcoholic fatty liver disease (NAFLD). Excessive energy consumption would enhance

39 lipid accumulation [8] and hepatic steatosis is found in most of the cases [9]. Substantial lipid
40 accumulation in the liver is a hallmark of NAFLD, which leads to subsequent development of
41 cirrhosis, liver cancer, and increased mortality [10]. The causal relationship between ID and
42 hepatic steatosis needs to be clarified.

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

51 In the literature, ID without anemia impairs cognitive function [14] and endurance capacity
52 [15,16] in clinical trials, as well as heart function [17] in a culture experiment. These
53 observations suggest that MID could possibly modulate cellular functions. Since the liver is
54 responsible for iron metabolism, a marginal reduction in iron concentration might influence
55 lipid metabolism in the liver. The aim of the present study was to establish diet-induced MID
56 in rats and to investigate whether MID influences liver lipid accumulation in combination
57 with dietary sucrose.

58

59 **Material & Methods**

60 *Animal experiments*

61 The study was approved by the Institutional Animal Care and Use Committee of National
62 Corporation Hokkaido University (approval number: 14-0026 and 17-0119), and all animals

63 were maintained in accordance with the Hokkaido University Manual for Implementing
64 Animal Experimentation. Male Wistar rats (3 weeks old; Japan SLC Inc., Hamamatsu, Japan)
65 were housed individually in a controlled environment at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity. The
66 light period was from 08:00 to 20:00. The rats had free access to food and water for the entire
67 study period. In the first experiment, the rats were acclimated on an AIN-93G-based iron
68 adequate (IA) diet [18] for 1 week and then divided into three dietary groups (Table 1), the
69 IA diet, the ID diet (iron-free), and the MID diet (IA:ID = 1:1, w/w). In the second
70 experiment, male Wistar rats (3 weeks old) were acclimated on the IA diet for 1 week and
71 then divided into four groups. Each group was fed one of four diets for 3 weeks as follows:
72 the IA diet, the MID diet, a high sucrose-based IA diet (HS-IA) and a HS-based MID diet
73 (HS-MID) (Table 2). In each experiment, body weight and food intake were measured every
74 day. The aortic blood plasma was collected under anesthesia with sodium pentobarbital (50
75 mg/kg body weight) into a syringe containing heparin (final concentration at 50 IU/mL) and
76 aprotinin (final concentration at 500 KIU/mL). Plasma was separated by centrifugation at
77 $2,000 \times g$ for 10 min at 4°C . After the rats were euthanized by exsanguination, the liver and
78 epididymal adipose tissue were collected and weighed. The collected plasma and tissues were
79 stored at -80°C until analysis. All rats were euthanized by exsanguination within three hours
80 (start from 10 AM). Food deprivation was not performed in all experiments.

81

82 ***Biochemical analysis***

83 For lipid extraction, 100 mg of liver was immersed in an extraction solution
84 (chloroform:methanol = 2:1) [19] for 2 days. The extracts were collected and evaporated in a
85 fume hood. Lipids in the extract were dissolved in 2-propanol for measurement. The
86 activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST)
87 were analyzed by using a transaminase CII-test Wako kit (Wako Pure Chemical Industries,

88 Osaka, Japan). Cholesterol and triglyceride levels were measured by using a cholesterol E-
89 test Wako kit (Wako) and triglyceride E-test Wako kit (Wako), respectively. The plasma
90 concentrations of iron, ferritin, glucose and insulin were determined by using an Iron Assay
91 kit LS - Ferrozine method (Metallogenics, Chiba, Japan), a Ferritin (Rat) ELISA Kit (Abnova,
92 Taipei, Taiwan), a Glucose CII-Test Wako (wako) and a LBIS Insulin-Rat-T (Shibayagi,
93 Gunma, Japan), respectively. Blood hemoglobin level was determined by using a
94 Hemoglobin Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA).

95

96 ***Measurement of liver glucose***

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

102

103 ***Liver iron analysis***

104 Liver iron was extracted as previously described [20] with minor modifications. Briefly,
105 the freeze-dried livers were weighed and milled to fine powder. The liver powder was dry-
106 ashed at 550°C for 30 h in a polarized muffle furnace (TMF-3200; Tokyo Rikakikai, Tokyo,
107 Japan) and heated with 20% nitric acid until evaporated, and a 3% nitric acid solution was
108 added to the ash. The iron concentration in the extracted samples was measured with an
109 atomic absorption spectrophotometer (Z-5310; Hitachi High-Technologies Corporation,
110 Tokyo, Japan).

111

112 ***Real-time quantitative polymerase chain reaction (RT-qPCR)***

113 Liver mRNA expression was measured using RT-qPCR. Total RNA was extracted using
114 an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions,
115 and the RNA concentration was measured with a NanoDrop Lite Spectrophotometer (Thermo
116 Scientific, Waltham, MA). The complementary DNA was synthesized from 1 µg of the RNA
117 using ReverTraAce® qPCR RT master mix with gDNA remover (Toyobo Co., Ltd., Osaka,
118 Japan) according to the manufacturer's instructions. The qPCR was performed using an
119 Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) with TaqMan Gene
120 Expression Assays (Rn03302271_gH for ribosomal protein lateral stalk subunit P0 (*Rplp0*),
121 Rn01495769_m1 for sterol regulatory element binding protein-1c (*Srebp1c*),
122 Rn01463550_m1 for fatty acid synthase (*Fas*), Rn00580702_m1 for carnitine
123 palmitoyltransferase 1 (*Cpt1*), and Rn00580432_m1 for interleukin-1 beta (*Il1β*) (Life
124 Technologies, Carlsbad, CA, USA). Relative expression levels were calculated for each
125 sample after normalization to those of *Rplp0* as a reference gene using the standard curve
126 method.

127

128 ***DNA extraction and analysis of mitochondrial DNA (mtDNA)***

129 Liver DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). The amount of
130 mtDNA and nuclear DNA (nDNA) was determined by real-time qPCR [21] with specific
131 primer pairs for mitochondrial gene encoded 16S RNA (*Mt-Rnr2*, forward: 5'-
132 ACTCGTTAGCCCAACACAGG-3', reverse: 5'-CCGTTTAACTTTAGTCACTG-3',
133 annealing temperature at 62°C, 152 bp) and nuclear gene encoded *Rplp0* (forward: 5'-
134 AGGGGCTTAGTCGAAGAGACC-3', reverse: 5'-GACAGTCCTTAACAGGAAGGGTA-
135 3', annealing temperature at 62°C, 236 bp). To determine the number of cells, we calculated
136 the ratio of the mtDNA against the nDNA as an index for the number of mitochondria per
137 cell. For PCR, 2 µl of sample DNA (5 ng/µl) was mixed with 0.25 µl of each primer (10 µM),

138 4.5 μ l of nuclease-free water, 0.25 μ l of ROX Reference Dye II marker, and 6.25 μ l of SYBR
139 Premix Ex Taq. The reaction was started at 95°C for 10 s, followed by 40 cycles of
140 denaturation at 95°C for 5 s, annealing at 61°C for 15 s, and extension at 72°C for 10 s.
141 Amplification curves were analyzed using an Mx3000P real-time PCR system (Stratagene) to
142 determine the mtDNA/nDNA ratio in each sample.

143

144 ***Statistical Analysis***

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

151

152 **Results**

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

154 To confirm whether the MID diet induces ID without anemia, we fed the rats with the
155 experimental diet for three weeks in the first experiment. A significant reduction in food
156 intake in ID-fed rats was detected from day 3 compared to that in the IA-fed rats (Fig. 1A),
157 and the cumulative food intake also decreased significantly in the ID-fed rats (284 ± 4 g)
158 compared with that in the IA-fed rats (326 ± 7 g). Similarly, a significant difference in body
159 weight was observed in the ID-fed rats compared with that in the IA-fed rats from day 13
160 (Fig. 1B), and the final body weight was significantly reduced in the ID-fed rats (213 ± 3 g)
161 compared to that in the IA rats (238 ± 3 g). These results are consistent with previous

162 observations in severe ID experiments [13]. Liver weight was significantly decreased in the
163 MID (4.1 ± 0.2 g/100 g body weight) and ID-fed rats (3.9 ± 0.2 g/100 g body weight)
164 compared to that in the IA-fed rats (4.4 ± 0.3 g/100 g body weight). Epididymal adipose
165 tissue weight was significantly reduced in the ID-fed rats (1.7 ± 0.3 g/100g body weight) but
166 not in the MID-fed rats (2.0 ± 0.3 g/100 g body weight) compared to that in the IA-fed rats
167 (2.1 ± 0.3 g/100 g body weight). The cumulative food intake and final body weight of the ID-
168 fed rats were 87.1% and 89.8% of those of the IA-fed rats, respectively. Notably, no
169 difference was found in daily food intake and body weight between the MID- and IA-fed rat
170 groups during the experiment. These results suggest that food consumption and growth in the
171 MID-fed rats were almost comparable with those in the IA-fed rats.

172

173 ***The MID diet induces ID without anemia***

174 To evaluate whether the MID diet reduced hepatic iron concentration without anemia, we
175 analyzed iron-related parameters (Fig. 2). The ID diet reduced iron concentrations in both
176 plasma and liver, accompanied by decreased hematocrit and hemoglobin levels as well as
177 plasma ferritin concentration. On the other hand, the MID diet decreased plasma ferritin and
178 iron concentrations in the plasma and liver as shown in Fig. 2A-C, but no induction of anemia
179 was observed in the MID-fed rats, judging by the hematocrit and hemoglobin levels (Fig. 2D,
180 E). Therefore, we determined that the MID diet successfully induces ID in the plasma and
181 liver without anemia. On the other hand, no difference was observed in liver triglyceride
182 concentration between the rats fed MID (13.2 ± 2.5 mg/g liver) and IA diets (12.6 ± 2.1 mg/g
183 liver).

184

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

186 As the consumption of the MID diet was still insufficient to induce hepatic lipid
187 accumulation in the first experiment, we hypothesized that some lipogenic stimuli are
188 required to cause lipid accumulation in MID. It has been reported that an increase in
189 expression of lipogenic genes in response to ID was observed on an AIN-76A diet but not on
190 an AIN-93 diet [22]. We chose a HS diet as a lipogenic-promoting diet [23,24] because the
191 major carbohydrate source in AIN-76A and AIN-93 is sucrose and starch, respectively [18].
192 As a result, no differences were observed in food intake, body weight, and epididymal
193 adipose tissue weight among all groups (Table 3). The HS diet significantly increased liver
194 weight (two-way ANOVA, $P < 0.0001$) regardless of the iron concentration in the diet. The
195 MID diet decreased liver iron concentration without a reduction in hemoglobin level (Fig.
196 3A-C), although a reduction in plasma iron concentration was found only with the control
197 diet in response to a decrease in dietary iron (Fig. 3B). These results suggest that the MID
198 diet can also induce hepatic ID in the HS diet. Intriguingly, the MID diet significantly
199 increased liver triglyceride concentration only in the HS-fed condition (Fig. 4A). Two-way
200 ANOVA analysis revealed a significant interaction between sucrose and iron in the values of
201 liver triglycerides. Although the HS diet significantly increased plasma TG, there was no
202 difference in the other parameters related to lipid metabolism (Fig. 4B-D). We determined
203 plasma transaminase activities (Fig.4E-F) as lipotoxicity markers associated with liver injury
204 [25]. Although HS significantly elevated plasma ALT, the alteration was not necessarily
205 associated with liver triglyceride concentration (Fig. 4F). No difference was observed in AST
206 activity (Fig. 4G) among the groups.

207

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

209 Severe ID was associated with an increase in the hepatic expression of lipogenic genes [12].
210 Thus, we measured expression levels of the genes related to lipid synthesis as well as

211 inflammation (Fig. 5). Notably, consistent with liver lipid accumulation, a significant
212 increase was found in *Fas* expression in the MID-fed rats in response to HS (Fig. 5A). In
213 contrast, no change was observed in the expression of *Srebp1c* and *Cpt1* (Fig. 5B-C). The HS
214 significantly increased aortic plasma glucose but not MID (Control: 6.9 ± 0.6 , MID: 7.2 ± 0.7 ,
215 HS: 9.1 ± 1.7 , HS+MID: 9.3 ± 1.6 , two-way ANOVA, $P = 0.0005$ for HS). No difference was
216 observed in liver glucose and aortic insulin level among the groups (data not shown). We also
217 measured interleukin-1 β (*Il1 β*) gene expression as a marker of inflammation, but no
218 difference was observed (Fig. 5D). We measured the relative amount of mtDNA as liver
219 mitochondria are the major organelle for fatty acid β -oxidation. As a result, the
220 mtDNA/nDNA ratio tended to decrease in response to a reduction in dietary iron
221 concentration (Fig. 5E, $P = 0.0807$).

222

223 **Discussion**

224 Reduction in hepatic iron concentration is expected to influence liver function in ID, even
225 in the absence of anemia. Previous studies have shown that hepatic lipid accumulation was
226 accompanied by anemia in the ID model [11]. However, the incidence of MID is more
227 widespread than severe ID [2]. The primary aim of this study was to establish a reasonable
228 MID model. The present study demonstrated that the diet containing a 50% concentration of
229 the iron content in the AIN-93 mineral mixture successfully induced a reduction in iron
230 concentration in the liver and plasma without anemia. In contrast, the ID diet induced anemia
231 as confirmed by the reduction in hemoglobin or hematocrit levels, which is in line with the
232 results of previous study [11]. The major influence of the ID diet is retarded growth with
233 reduction in food intake [13]. Importantly, the present study demonstrated no influence of the
234 MID diet on voluntary food intake and growth. In humans, we do not usually notice and are

235 not able to evaluate the precise iron consumption rate in daily life, which might increase the
236 prevalence of ID without anemia. The MID-fed rats are considered to reflect presymptomatic
237 conditions in human ID without anemia.

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

255 Liver lipid accumulation is considered to be mainly due to an imbalance of de novo
256 lipogenesis and fatty acid catabolism. Because Fas catalyzes the terminal step in the
257 biogenesis of fatty acids, *Fas* expression is thought to be a marker of lipogenesis. We found
258 that enhancement of *Fas* expression was closely associated with liver triglyceride
259 accumulation in the HS-MID-fed rats, suggesting that liver ID promoted liver triglyceride

260 accumulation partially via enhancement of *Fas* gene expression in the presence of sucrose.
261 Interestingly, levels of *Srebp1c*, a major positive regulator of *Fas* expression [38,39], failed
262 to increase in each treatment, suggesting that *Srebp1c* expression was not responsible for the
263 increase in *Fas* expression in the HS-MID-fed rats. Glucose is the major ligand for another
264 key regulator in the expression of the *Fas* gene, carbohydrate-responsive element binding
265 protein (Chrebp) [40]. Several reports have been suggested that ID animals utilize
266 carbohydrate as an energy source instead of fat [12,41–43]. Additionally, an increase in
267 hepatic glucose production has been reported in ID anemic rats [41]. Hepatic iron depletion
268 by deferoxamine, an intracellular iron chelator, enhances hepatic glucose uptake [44].
269 However, the MID did not affect liver glucose and aortic insulin levels although the HS
270 significantly increased aortic plasma glucose level, suggesting that liver ID by the MID diet
271 is not sufficient to impair glucose metabolism. These results should be carefully interpreted
272 because we did not perform fasting before collecting the samples. Alternatively, it has been
273 reported that replacement of dietary glucose with fructose further increases Chrebp activity
274 [45]. It is possible that liver ID and fructose synergistically activate Chrebp, resulting in an
275 upregulation of *Fas* gene expression because of the pivotal difference in dietary fructose level
276 between the control diet and the HS diet.

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

284 Free fructose and sucrose appear to have a similar pathological effect [47] and fructose is
285 considered to be more lipogenic than glucose [32, 48]. In humans, average fructose intake
286 was estimated at 49 g/day [49], which is equivalent to 196 kcal/day. As the energy intake of
287 human adults is 2,000–2,500 kcal/day, daily energy intake from fructose (about 200 kcal/day)
288 estimated to be at 8-10%. In the present study, the rats fed the HS diet appear to consume
289 quite a high level of fructose (32% of their total energy intake). However, it is common to use
290 diet containing a high dose of fructose (~66%) to develop metabolic syndrome in a short
291 period [50]. In addition, rodents may have resistance against fructose ingestion than humans.
292 Jang et al. [51] demonstrated that small intestine initially metabolizes ingested fructose rather
293 than liver in mice. Indeed, ingestion of fructose (0.25 g/kg, as a 1:1 mixture of glucose and
294 fructose) in mice would not appear in portal blood as fructose [51]. In contrast, in humans,
295 ingestion of a lower dose of fructose (0.1–0.15 g fructose/kg if body weight was 50–75 kg, as
296 a 10:1 mixture of glucose and fructose) increased in circulating fructose concentration nearly
297 two-fold [52]. Hence, rodent liver may be less sensitive to fructose ingestion than humans
298 due to high metabolic capacity of the small intestine as some researchers proposed [51, 53].
299 Furthermore, there are some reports showing that humans consume a high level of dietary
300 fructose as follows. In NAFLD patients, consumption of fructose is more than twofold higher
301 than healthy individuals, amounting to 90 g from sweetened beverages alone [54]. Ventura et
302 al. [55] demonstrated that high fructose corn syrup (HFCS), a major source of fructose in
303 sweetened beverages, contains higher fructose amount than that of disclosures from
304 producers. They assumed actual human fructose intake may be around 18% higher than
305 previously estimated by Marriott and colleagues [49].

306 In conclusion, we established ID without anemia by ingestion of the MID diet and found
307 an increase in hepatic lipid accumulation in the MID-fed rats in the presence of large amounts
308 of dietary sucrose accompanied by the enhancement of *Fas* gene expression. Epidemiological

309 studies showed the prevalence of inadequate iron status [56] and an inverse correlation
310 between hepatic iron concentration and hepatic steatosis in NAFLD patients [57]. The present
311 study suggests that liver-specific ID in the presence of enormous fructose triggers hepatic
312 lipid accumulation and that iron is a potent modulator for preventing NAFLD.

313

314 **Author Contribution**

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

317

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321

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454

455 **Figure legends**

456 ***Fig. 1. Changes in food intake and body weight in the rats fed the IA, MID, and ID diets***
457 ***during the experiment.***

458 (A) Food intake, (B) Body weight. Data are presented as the mean \pm SEM. Asterisk
459 represents significant differences from the value of the IA group at each time point ($P < 0.05$,
460 $n = 8$).

461

462 ***Fig. 2. Iron-related parameters in the rats fed the IA, MID, and ID diets for 3 weeks.***

463 (A) Liver iron, (B) Plasma iron, (C) Plasma ferritin, (D) Hematocrit, (E) Hemoglobin. Data
464 are presented as the mean \pm SEM. Asterisk represents significant differences from the value
465 of the IA-fed rats ($P < 0.05$, $n = 8$).

466

467 ***Fig. 3. Iron-related parameters in the rats fed the MID diet with or without HS for 3 weeks***

468 (A) Liver iron, (B) Plasma iron, (C) Hemoglobin in the rats fed control or the HS diet
469 combined with either IA or MID diet for 3 weeks. Data are presented as the mean \pm SEM.

470

471 ***Fig. 4. Liver lipids, plasma lipids, and plasma transaminase activity in the rats fed the HS-***

472 ***MID diet for 3 weeks***

473 (A) Liver triglyceride (TG) concentration, (B) Plasma TG concentration, (C) Liver
474 cholesterol (Cho), (D) Plasma Cho concentration, (E) Plasma ALT activity and (F) Plasma
475 AST. White and gray bars represent the data of the rats fed the IA and MID diets,
476 respectively. Values are presented as the mean \pm SEM. Asterisk represents significant
477 differences from the data of the IA-fed rats and the HS-fed rats ($P < 0.05$, $n = 6$).

478

479 **Fig. 5. Gene expression and mtDNA content in the liver of the rats fed the HS-MID diet for**
480 **3 weeks.**

481 Gene expression levels were measured in fatty acid synthase (*Fas*) (A), sterol regulatory
482 element binding protein 1 (*Srebp1*) (B), carnitine palmitoyltransferase 1 (*Cpt1*) (C), and
483 interleukin-1 β (*Il1 β*) (D). The target gene expression levels were normalized to *Rplp0* mRNA
484 expression. Liver mitochondrial content (E) was normalized to the nuclear DNA content.
485 White and gray bars represent the data of the rats fed the IA- and MID-diets, respectively.
486 Values are presented as the mean \pm SEM. Asterisk represents significant differences from
487 data of the IA-fed rats and the HS-fed rats ($P < 0.05$, n = 6).

488

489

490 **Tables**

491 Table1. Diet composition in the first experiment

492		IA	MID	ID
493		g/kg diet		
494	Casein ¹	200	200	200
495	Dextrin ²	529.5	529.5	529.5
496	Sucrose ³	100	100	100
497	Soybean oil ⁴	70	70	70
498	Cellulose ⁵	50	50	50
499	Mineral mixture for IA ⁶	35	-	-
500	Mineral mixture for ID ⁷	-	-	35
501	Mineral mixture for MID ⁸	-	35	-
502	Vitamin mixture ⁹	10	10	10
503	L-Cystine ¹⁰	3	3	3
504	Choline hydrogen tartrate ¹⁰	2.5	2.5	2.5

505 ¹ NZMP Acid Casein (Fonterra Co-Operative Group Limited, Auckland, New Zealand),

506 ² TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

507 ³ Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan

508 ⁴ J-Oil Mills, Inc., Tokyo, Japan

509 ⁵ Crystalline cellulose (Ceolus PH-102, Asahi Kasei Chemicals Corp., Tokyo, Japan)

510 ⁶ AIN-93G Mineral mixture [29]

511 ⁷ The mineral mixtures for ID (iron-free) were formulated by adding sucrose at the expense of ferric
512 citrate from the mineral mixture for IA

513 ⁸ The mineral mixtures for MID were formulated by mixing the mineral mixtures for IA and ID
514 (IA:ID = 1:1, w/w)

515 ⁹ AIN93 vitamin mixture (MP Biomedicals, USA)

516 ¹⁰ Wako Pure Chemical Industries, Ltd., Osaka, Japan

517 Table2. Diet composition in the second experiment

518		Control		HS	
519		IA	MID	IA	MID
520		g/kg diet			
521	Casein ¹	200	200	200	200
522	Dextrin ²	529.5	529.5	-	-
523	Sucrose ³	100	100	629.5	629.5
524	Soybean oil ⁴	70	70	70	70
525	Cellulose ⁵	50	50	50	50
526	Mineral mixture for IA ⁶	35	-	35	-
527	Mineral mixture for MID ⁷	-	35	-	35
528	Vitamin mixture ⁸	10	10	10	10
529	L-cystine ⁹	3	3	3	3
530	Choline Hydrogen Tartrate ⁹	2.5	2.5	2.5	2.5

531 ¹ NZMP Acid Casein (Fonterra Co-Operative Group Limited, Auckland, New Zealand),

532 ² TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

533 ³ Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan

534 ⁴ J-Oil Mills, Inc., Tokyo, Japan

535 ⁵ Crystalline cellulose (Ceolus PH-102, Asahi Kasei Chemicals Corp., Tokyo, Japan)

536 ⁶ AIN-93G mineral mixture [29]

537 ⁷ The mineral mixtures for MID were exactly the same composition as shown in Table 1

538 ⁸ AIN93 vitamin mixture (MP Biomedicals, USA)

539 ⁹ Wako Pure Chemical Industries, Ltd., Osaka, Japan

540

541 Table3. Growth parameters and tissue weight in rats of the second experiment

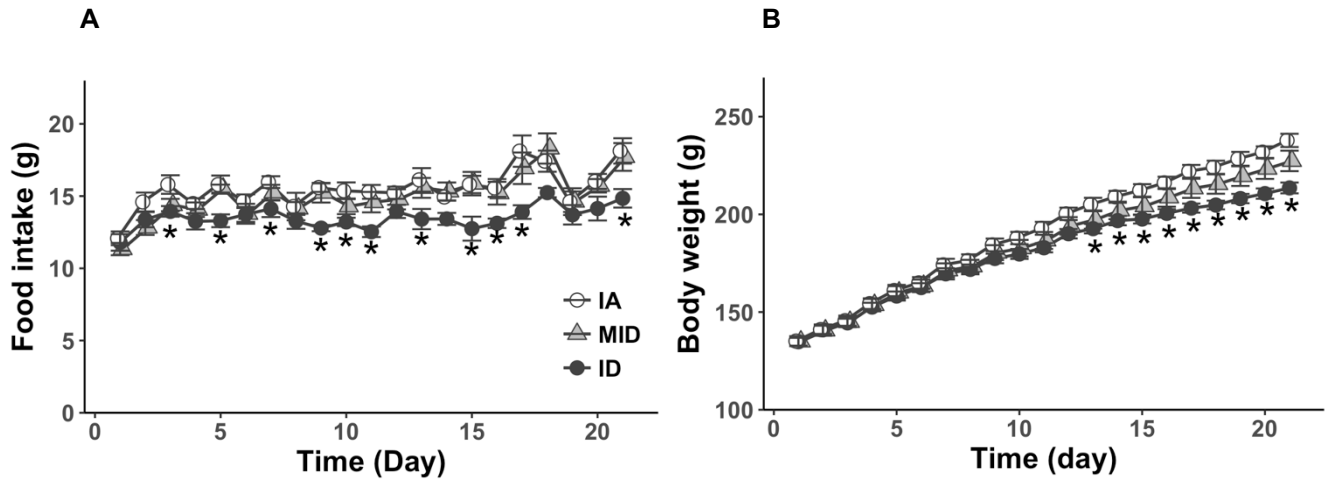
542	Control		HS		Two-way ANOVA, <i>P</i> value			
543	IA	MID	IA	MID	Iron (I)	Sucrose (S)	I × S	
544								
545	Growth parameters (g)							
546	Initial body weight	121 ± 2	121 ± 3	121 ± 4	122 ± 3	NS	NS	NS
547	Total food intake	287 ± 13	310 ± 11	301 ± 8	294 ± 5	NS	NS	NS
548	Final body weight	228 ± 7	232 ± 6	229 ± 4	225 ± 4	NS	NS	NS
549	Tissue weight							
550	(g 100g body weight)							
551	Liver	4.50 ± 0.1	4.30 ± 0.9	5.04 ± 0.2	4.89 ± 0.1	NS	<0.0001	NS
552	Epididymal adipose tissue	1.93 ± 0.1	1.84 ± 0.1	1.86 ± 0.1	1.80 ± 0.8	NS	NS	NS
553	Means values with their standard errors.							

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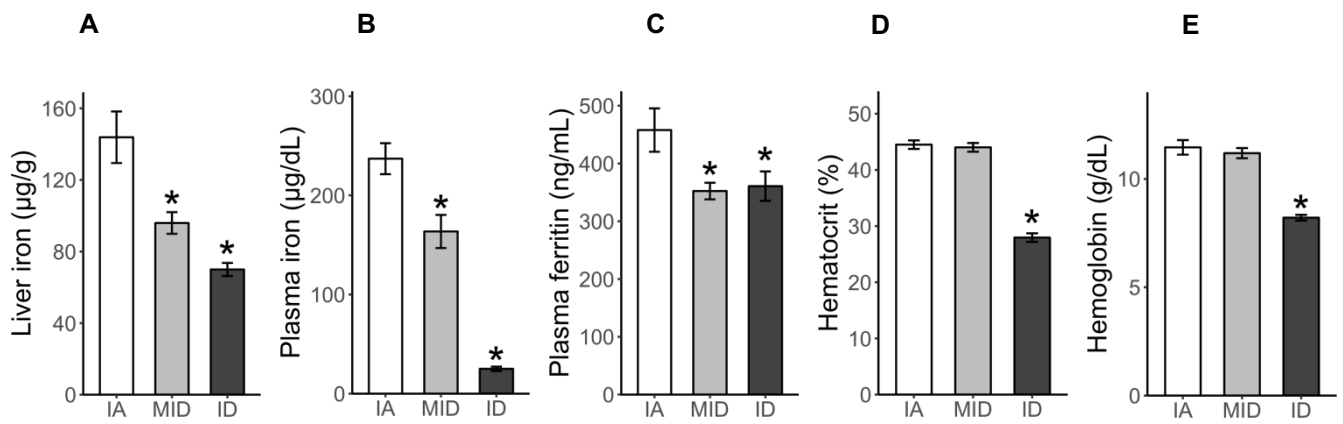
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Figure 1



50 **Figure 2**

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69 **Figure 3**

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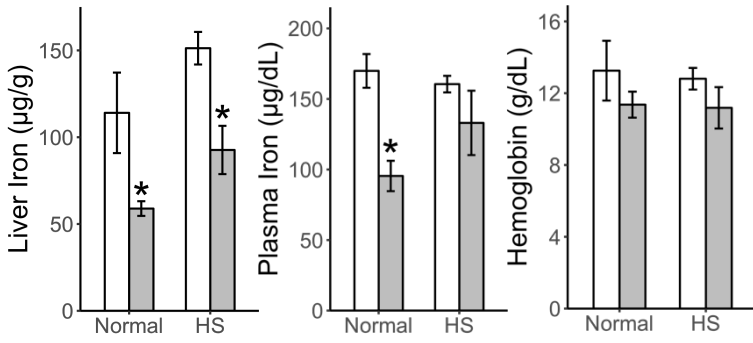
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C



Two-way ANOVA, p value	Iron (I)	Sucrose (S)	I × S
Liver iron	0.0008	0.0234	NS
Plasma iron	0.0019	NS	NS
Hemoglobin	NS	NS	NS

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114 **Figure 4**

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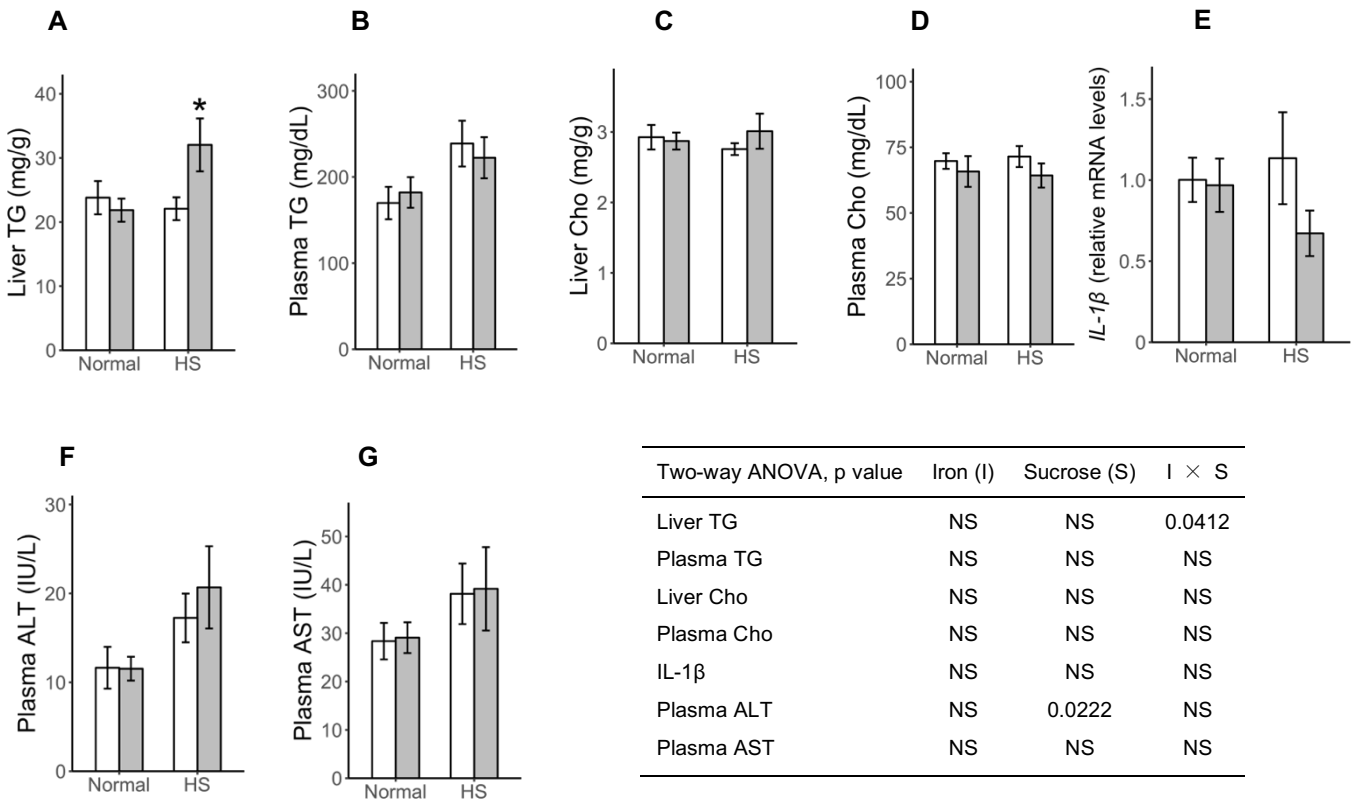
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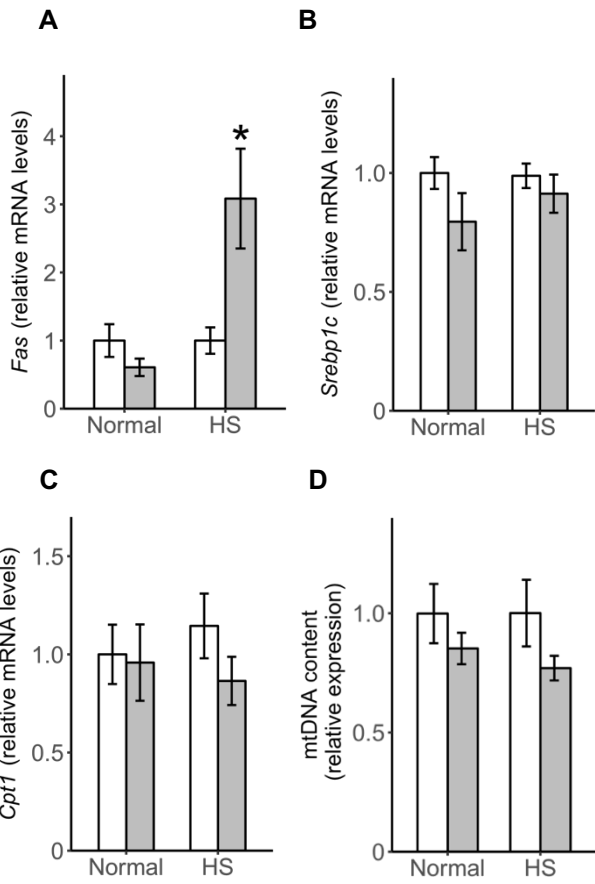
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Two-way ANOVA, p value	Iron (I)	Sucrose (S)	I × S
Fas	0.0483	0.0059	0.0060
Srebp1c	NS	NS	NS
Cpt1	NS	NS	NS
mtDNA	0.0807	NS	NS

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