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Effect of Korean pine nut oil on hepatic iron, copper, and zinc status and expression of genes and proteins related to iron absorption in diet-induced obese mice

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ABSTRACT

Purpose: Body adiposity is negatively correlated with hepatic iron status, and Korean pine nut oil (PNO) has been reported to reduce adiposity. Therefore, we aimed to study the effects of PNO on adiposity, hepatic mineral status, and the expression of genes and proteins involved in iron absorption.
Methods: Five-week-old male C57BL/6 mice were fed a control diet containing 10% kcal from PNO (PC) or soybean oil (SBO; SC), or a high-fat diet (HFD) containing 35% kcal from lard and 10% kcal from PNO (PHFD) or SBO (SHFD). Hepatic iron, copper, and zinc content; and expression of genes and proteins related to iron absorption were measured.
Results: HFD-fed mice had a higher white fat mass (2-fold; $p < 0.001$), lower hepatic iron content (25% lower; $p < 0.001$), and lower hepatic *Hamp* ($p = 0.028$) and duodenal *Dcytb* mRNA levels ($p = 0.037$) compared to the control diet-fed mice. Hepatic iron status was negatively correlated with body weight ($r = -0.607$, $p < 0.001$) and white fat mass ($r = -0.745$, $p < 0.001$). Although the PHFD group gained less body weight (18% less; $p < 0.05$) and white fat mass (18% less; $p < 0.05$) than the SHFD group, the hepatic iron status impaired by the HFD feeding did not improve. The expression of hepatic and duodenal ferroportin protein was not affected by the fat amount or the oil type. PNO-fed mice had significantly lower *Slc11a2* ($p = 0.022$) and *Slc40a1* expression ($p = 0.027$) compared to SBO-fed mice. However, the PC group had a higher *Heph* expression than the SC group ($p < 0.05$). The hepatic copper and zinc content did not differ between the four diet groups, but hepatic copper content adjusted by body weight was significantly lower in the HFD-fed mice compared to the control diet-fed mice.
Conclusion: HFD-induced obesity decreased hepatic iron storage by affecting the regulation of genes related to iron absorption; however, the 18% less white fat mass in the PHFD group was not enough to improve the iron status compared to the SHFD group. The hepatic copper and zinc status was not altered by the fat amount or the oil type.

Keywords: dietary fats, pinolenic acid, high-fat diet, obesity, trace elements

Conflict of Interest
There are no financial or other issues that might lead to conflict of interest.

INTRODUCTION

Obesity has been reported to disturb iron homeostasis in both human subjects [1-3] and animal models [4-6]. Non-anemic iron deficiency was observed in obese postmenopausal women [1], and overweight or obese adolescents had the higher prevalence of iron deficiency than normal weight adolescents [2]. High-fat diet (HFD) feeding significantly reduced hepatic non-heme iron status and iron-absorption related gene expression in mice [4,5]. A 6-month weight loss program improved iron absorption and storage in obese children and adolescents [3], and obesity-induced depletion of hepatic iron was restored by low-fat diet feeding in mice [6]. These results suggest that adiposity is a major factor contributing to iron deficiency.

Other divalent cations, including zinc and copper, compete with iron for transporters, including divalent metal transporter 1 (DMT1), and other uptake mechanisms due to the chemical similarities [7-9]. Human adults supplemented with zinc combined with ferrous iron had significantly lower plasma zinc level compared to those supplemented with zinc only [10], and human infants fed formula with higher iron concentration had reduced copper absorption [11]. In addition, *ob/ob* mice were reported to have lower hepatic copper and zinc levels adjusted by hepatic triglyceride content than lean mice [12]. These reports suggest a possibility that obesity may dysregulate zinc and copper metabolism as well as iron metabolism; however, the effects of HFD on zinc and copper status has not been well studied.

Korean pine nut oil (PNO) has been reported to exert multiple health benefits, and pinolenic acid (18:3, Δ5,9,12), a unique fatty acid found in PNO, is considered as a key factor for the beneficial effect [13-17]. In a clinical study, PNO was shown to increase the release of satiety hormone and decrease the appetite [13]. It has been also reported that PNO reduced weight gain [14], increased adipose thermogenesis [15], suppressed intestinal lipid uptake [16], and attenuated hepatic lipid accumulation in HFD-fed mice [17]. These health benefits of PNO suggest that substitution of dietary fat with PNO could reverse obesity-induced dysregulation of trace mineral absorption and storage. Therefore, we hypothesized that PNO reduces adiposity and reverses the lower hepatic mineral status induced by HFD feeding. To test the hypothesis, we investigated the effects of PNO on body fat accumulation, hepatic mineral status, and expression of genes and proteins related to iron absorption in control diet- or HFD-fed mice.

METHODS

Animals and diets

Five-week-old male C57BL/6 mice were purchased from Central Laboratory Inc (Seoul, Korea) and kept in a specific pathogen-free animal care facility at Seoul National University. Animals were acclimated for 3 days before they were assigned to experimental diets. To determine the effects of PNO on adiposity and hepatic mineral status, soybean oil (SBO) was used as the control oil. This is because SBO is one of the most consumed oil in the world, and as we reported before [14,17], the fatty acid composition of SBO is similar to that of PNO (**Supplementary Table 1**). Animals were fed for 12 weeks with control diets containing 10% kcal fat from SBO (SC) or PNO (PC), or high fat diets (45% kcal fat, HFD) containing 35% kcal fat from lard and 10% kcal fat from SBO (SHFD) or PNO (PHFD). PNO, a gift from Dubio Co., Ltd. (Hwaseong, Korea), was sent to Dyets Inc. (Bethlehem, PA, USA) to make experimental diets. The composition of experimental diets is shown in **Table 1**. The estimated

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Table 1. Composition of the experimental diets (g)

Ingredients	SC	SHFD	PC	PHFD
Casein	200	200	200	200
L-Cystine	3	3	3	3
Sucrose	350	172.8	350	172.8
Cornstarch	315	72.8	315	72.8
Dyetrose[1)]	35	100	35	100
PNO	0	0	45	45
SBO	45	45	0	0
Lard	0	157.5	0	157.5
t-Butylhydroquinone	0.009	0.009	0.009	0.009
Cellulose	50	50	50	50
Mineral Mix[2)]	35	35	35	35
Vitamin Mix[3)]	10	10	10	10
Choline Bitartrate	2	2	2	2
Total	1,045.0	848.1	1,045.0	848.1
kcal/g diet	3.69	4.64	3.69	4.64

Resource: Dyets, Inc, Bethlehem, PA, USA.
SC group, control diet containing 10% kcal from SBO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil.
[1)]Dyetrose is dextrinized cornstarch containing 90–94% tetrasaccharides.
[2)]Thirty-five grams of mineral mix (Research Diets, Inc, New Burnswick, NJ, USA, S10026) provides 1.0 g sodium, 1.6 g chloride, 0.5 g magnesium, 0.33 g sulfur, 59 mg manganese, 45 mg iron, 29 mg zinc, 6 mg copper, 2 mg chromium, 1.6 mg molybdenum, 0.16 mg selenium, 0.9 mg fluoride, 0.2 mg iodine and 3.99 g sucrose.
[3)]Ten grams of vitamin mix (Research Diets, Inc, V10001) provides 4,000 IU vitamin A, 1,000 IU vitamin D_3, 50 IU vitamin E, 30 mg niacin, 16 mg pantothenic acid, 7 mg vitamin B_6, 6 mg vitamin B_1, 6 mg vitamin B_2, 2 mg folic acid, 0.5 mg menadione, 0.2 mg biotin, 10 ug vitamin B_{12} and 9.78 g sucrose.

iron, copper, and zinc contents are 43.06 mg Fe/kg diet, 5.74 mg Cu/kg diet, and 27.75 mg Zn/kg diet in the control diet, and 53.06 mg Fe/kg diet, 7.07 mg Cu/kg diet, and 34.19 mg Zn/kg diet in the HFD. Animals were given free access to autoclaved water and experimental diets, and maintained in a controlled temperature (23 ± 3°C), humidity (55 ± 10%), and 12-hour dark/light cycle. Body weight was measured weekly, and food intake was measured 4 times a week. After 12 weeks of feeding experimental diets, animals were fasted for 12 hours and euthanized by CO_2 asphyxiation. White adipose tissue (inguinal subcutaneous, epididymal, perirenal, and retroperitoneal depots), liver, and duodenum samples were collected, immediately frozen in liquid nitrogen, and stored at −80°C until subsequent analyses. All animal procedures were approved by the Animal Care and Use Committee at Seoul National University (Approval No. SNU-101029-1).

Determination of hepatic iron, copper, and zinc status

Wet ashing digestion was used to determine hepatic trace mineral status. Liver tissues were digested with 16N HNO_3 (Junsei, Tokyo, Japan) in a beaker placed on a hot plate at 60°C for 5 hours, and the digested samples were diluted into 10 mL of 0.1 N HNO_3. The concentrations of iron, copper, and zinc were measured by 210/211 VGP atomic spectrophotometer (Buck Scientific Inc, OH, USA). All plastic and glassware were soaked in 8N HNO_3 and rinsed with distilled water prior to use.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from liver and duodenum samples using Trizol (Invitrogen, CA, USA), and complementary DNA was synthesized using a PrimeScript™II 1st strand cDNA synthesis kit (Takara, Shiga, Japan). The mRNA levels of hepatic *Hamp* (Hepcidin encoding gene), and duodenal *Dcytb* (Duodenal cytochrome b reductase encoding gene), *Slc11a2* (DMT1

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Table 2. Primer sequences used for quantitative real-time polymerase chain reaction analysis

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Hamp	TTGCGATACCAATGCAGAAGA	GATGTGGCTCTAGGCTATGTT
Dcytb	GCAGCGGGCTCGAGTTTA	TTCCAGGTCCATGGCAGTCT
Slc11a2	GGCTTTCTTATGAGCATTGCCTA	GGAGCACCCAGAGCAGCTTA
Slc40a1	TTGCAGGAGTCATTGCTGCTA	TGGAGTTCTGCACACCATTGAT
Heph	TTGTCTCATGAAGAACATTACAGCAC	CATATGGCAATCAAAGCAGAAGA
Actb	ACCCACACTGTGCCCATCTA	CACGCTCGGTCAGGATCTTC

Hamp, hepcidin; *Dcytb*, duodenal cytochrome b reductase; *Slc11a2*, divalent metal transporter 1; *Slc40a1*, ferroportin; *Heph*, hephaestin; *Actb*, β-actin.

encoding gene), *Slc40a1* (Ferroportin encoding gene), and *Heph* (Hephaestin encoding gene) were quantified using a SYBR Premix Ex Taq (Takara, Shiga, Japan) and a StepOne Real-time PCR System (Applied Biosystems, CA, USA). The reaction was performed in duplicate, and the values were normalized to the mRNA expression of *Actb* (housekeeping β-actin encoding gene). The sequence of the primers is listed in **Table 2**.

Western blot analysis

Protein was extracted from liver and duodenum samples using RIPA buffer with 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined by Bradford method. Protein lysates (30 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad Laboratories, CA, USA). After blocking with 1X TBST containing 5% non-fat dry milk for 1 hour, membrane was incubated with anti-mouse MTP1 (Alpha Diagnostics International, TX, USA; MTP1 is also known as Ferroportin) diluted as 1:1000 in 1X TBST with 5% non-fat dry milk at 4°C overnight, and then incubated with secondary anti-rabbit IgG HRP-linked antibody (Cell signaling Technology, MA, USA) diluted as 1:1000 in 1X TBST with 5% non-fat dry milk at room temperature for 1 hour. To detect the signal, chemiluminescent assays using horseradish peroxidase (HRP) enzyme were conducted, and the signal was captured on X-ray film. The band intensities were quantified using Gel Doc XR system (Bio-Rad, CA, USA) and Quantity One 1-D analysis software (Bio-Rad, CA, USA).

Statistical analysis

Statistical analyses were carried out using SPSS (SPSS version 18.0; SPSS Inc., IL, USA). To determine the overall effects of fat amount, oil type, and the interaction, two-way analysis of variance (ANOVA) was conducted followed by the Fisher's least significance difference (LSD) post-hoc test for individual group comparisons. If data were not normally distributed, data transformation was conducted before ANOVA analyses. When the interaction between fat amount and oil type exists, Student's t-test was used to determine the difference between groups instead of using p-value of overall effects. The significant association between two variables was determined by Pearson correlation test. The value of $p < 0.05$ is considered as statistical significance. All data were expressed as mean ± standard error of the mean (SEM).

RESULTS

Body weight, tissue weight, and food intake

Overall, weight gain and white fat mass were significantly affected by both fat amount and oil type (**Table 3**). Mice fed HFD gained significantly more weight ($p < 0.001$) and white fat mass

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Table 3. Body weight and tissue weight of the mice

Parameters	SC (n = 10)	PC (n = 11)	SHFD (n = 11)	PHFD (n = 11)	p-value		
					Fat amount	Oil type	Interaction
Body weight at 0 wk (g)	17.30 ± 0.51	16.74 ± 0.45	17.01 ± 0.36	17.04 ± 0.34	0.97	0.56	0.50
Body weight at 12 wk (g)	32.48 ± 0.96^{ab}	30.51 ± 0.64^{a}	38.49 ± 1.45^{c}	34.58 ± 1.42^{b}	0.00	0.01	0.50
Body weight gain (g)	15.18 ± 0.84^{ab}	13.76 ± 0.59^{a}	21.48 ± 1.42^{c}	17.53 ± 1.31^{b}	0.00	0.01	0.32
Liver weight (g)	1.18 ± 0.04^{b}	1.09 ± 0.03^{ab}	1.21 ± 0.06^{b}	1.01 ± 0.04^{a}	0.55	0.00	0.29
Liver weight (g/g body weight)	0.38 ± 0.00^{b}	0.38 ± 0.00^{a}	0.33 ± 0.00^{a}	0.31 ± 0.00^{a}	0.00	0.26	0.18
Liver triglyceride (mg/g liver tissue)	29.25 ± 2.26	31.43 ± 1.91	39.50 ± 1.91	29.26 ± 1.98**	0.08	0.09	0.01
White adipose tissue (g)	3.10 ± 0.22^{b}	2.18 ± 0.18^{a}	5.34 ± 0.38^{c}	4.38 ± 0.39^{d}	0.00	0.00	0.94

Values are presented as mean ± SEM. Two-way analysis of variance was used to determine significant effects of fat amount and oil type followed by LSD post-hoc test for individual group comparisons. Different letters indicate statistical significance ($p < 0.05$). When the interaction effect was significant, Student's t-test was used to determine the difference between groups ($^{**}p < 0.01$). WAT includes inguinal subcutaneous, epididymal, perirenal, and retroperitoneal WAT.
SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil; WAT, white adipose tissue.

($p < 0.001$) than those fed the control diets. Mice in the PHFD group had significantly less weight gain (18% less, $p < 0.05$) and less amount of WAT (18% less, $p < 0.05$) than those in the SHFD group.

PNO-fed mice had lower liver weight than SBO-fed mice ($p < 0.001$), but when the liver weight was adjusted by body weight, there was no difference between the two (**Table 3**). HFD-fed mice had lower liver weight adjusted by body weight than control diet-fed mice ($p < 0.001$).

HFD contained 23% higher amount of iron, copper, and zinc than control diets, and mice fed HFD consumed approximately 13% less diet (g) than those fed control diets. Therefore, the mineral intake was slightly higher in the HFD group. There was no difference in daily food intake (g), daily energy intake (kcal), and daily mineral intake between SBO- and PNO-fed mice (**Table 4**).

Hepatic iron, copper, and zinc levels

Hepatic iron content was affected by fat amount, but not by oil type. Although HFD-fed mice had higher mineral intake than control diet-fed mice (**Table 4**), hepatic iron content was significantly lower in the HFD-fed mice compared to the control diet-fed mice. The SHFD group had 24% less amount of hepatic iron than the SC group ($p < 0.001$) and the PHFD group had 28% less than the PC group ($p < 0.001$). There was no difference in hepatic iron content between SHFD and PHFD groups despite the lower body weight and fat mass of the PHFD group, but hepatic iron content showed a strong negative correlation with body weight ($r = -0.607$, $p < 0.001$) and white fat mass ($r = -0.745$, $p < 0.001$, **Fig. 1**).

Table 4. Daily food and mineral intake of the mice

Parameters	SC (n = 10)	PC (n = 11)	SHFD (n = 11)	PHFD (n = 11)	p-value		
					Fat amount	Oil type	Interaction
Food intake (g/day)	3.20 ± 0.06^{b}	3.20 ± 0.03^{b}	2.82 ± 0.05^{a}	2.76 ± 0.04^{a}	0.00	0.54	0.48
Energy intake (kcal/day)	11.80 ± 0.21^{a}	11.82 ± 0.11^{a}	13.11 ± 0.22^{b}	12.82 ± 0.20^{b}	0.00	0.50	0.43
Fe intake (μg/day)	130.09 ± 2.32^{a}	130.34 ± 1.24^{a}	141.39 ± 2.41^{b}	138.32 ± 2.20^{b}	0.00	0.50	0.43
Cu intake (μg/day)	18.36 ± 0.33^{a}	18.40 ± 0.17^{a}	19.95 ± 0.34^{b}	19.52 ± 0.31^{b}	0.00	0.50	0.43
Zn intake (μg/day)	77.06 ± 1.37^{a}	77.20 ± 0.73^{a}	83.80 ± 1.43^{b}	81.98 ± 1.30^{b}	0.00	0.50	0.43

Values are presented as mean ± SEM. Two-way analysis of variance was used to determine significant effects of fat amount and oil type followed by LSD post-hoc test for individual group comparisons. Different letters indicate statistical significance ($p < 0.05$).
SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil.

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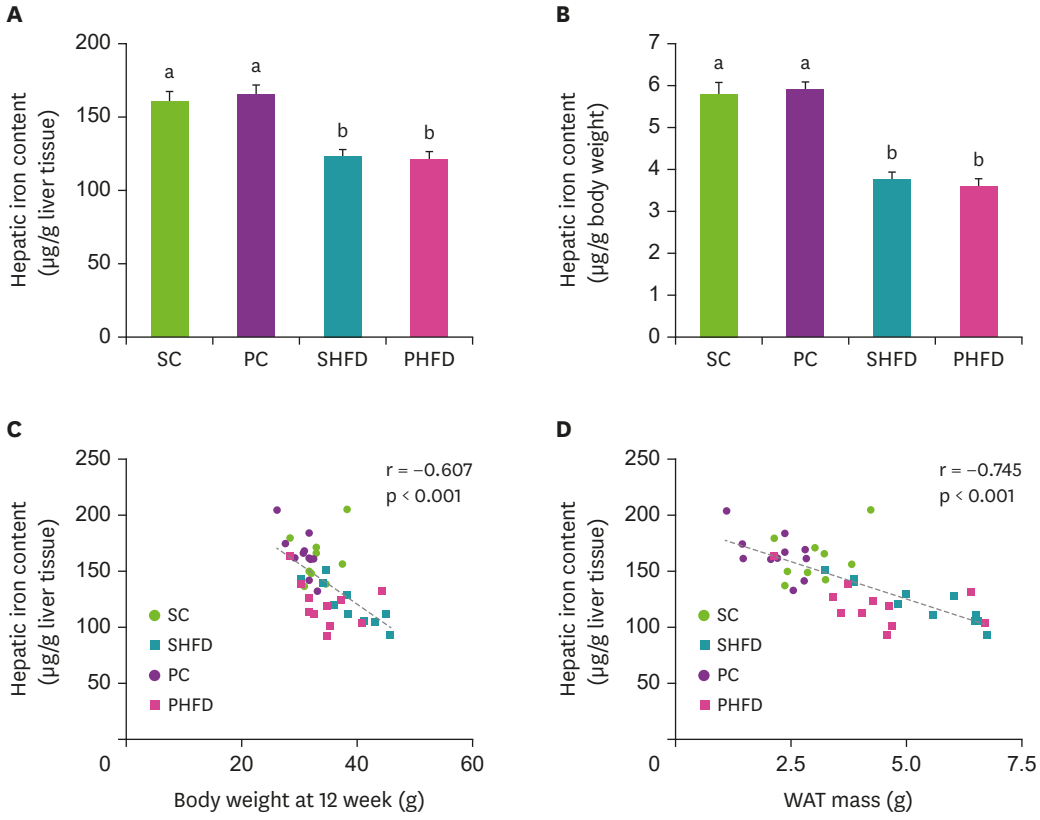

Fig. 1. Hepatic iron contents (A), hepatic iron adjusted by body weight (B), and the correlation between hepatic iron content and the body weight (C) or the WAT mass (D). Values are expressed as mean ± SEM (n = 10–11 per group). Two-way analysis of variance followed by LSD post-hoc test was performed, and different letters indicate statistical significance ($p < 0.05$). To determine the correlation between hepatic iron content and body weight or WAT mass, Pearson correlation test was conducted. WAT includes inguinal subcutaneous, epididymal, perirenal, and retroperitoneal depots.
SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil; WAT, white adipose tissue.

Neither the fat amount nor the oil type exerted a significant impact on hepatic copper and zinc content. However, hepatic copper level adjusted by body weight was significantly lower in mice fed HFD than those fed control diets (**Fig. 2**).

Hepcidin and Ferroportin expression

To test if the lower hepatic iron status in HFD-fed mice (both SHFD and PHFD groups) is caused by alterations in the expression of Hepcidin (a key hormone regulating iron homeostasis; encoded by *Hamp* gene) and Ferroportin (an iron exporter post-translationally regulated by hepcidin), *Hamp* mRNA expression in the liver (**Fig. 3**) and Ferroportin protein expression in the liver and duodenum were measured (**Fig. 4**). HFD-fed mice had lower hepatic *Hamp* expression ($p = 0.028$) than control-diet fed mice, and the PHFD group had significantly lower expression of *Hamp* in the liver than the PC group ($p = 0.025$). However, when the data was adjusted by the amount of hepatic iron storage, no effect of the fat amount and the oil type was observed. Both hepatic and duodenal Ferroportin protein expression levels were not different among the 4 different diet groups.

Iron absorption-related gene expression

To determine the effects of the 4 different experimental diets on intestinal iron uptake, the mRNA expression of duodenal *Dcytb* (an enzyme reducing Fe^{3+} to Fe^{2+} for the uptake via

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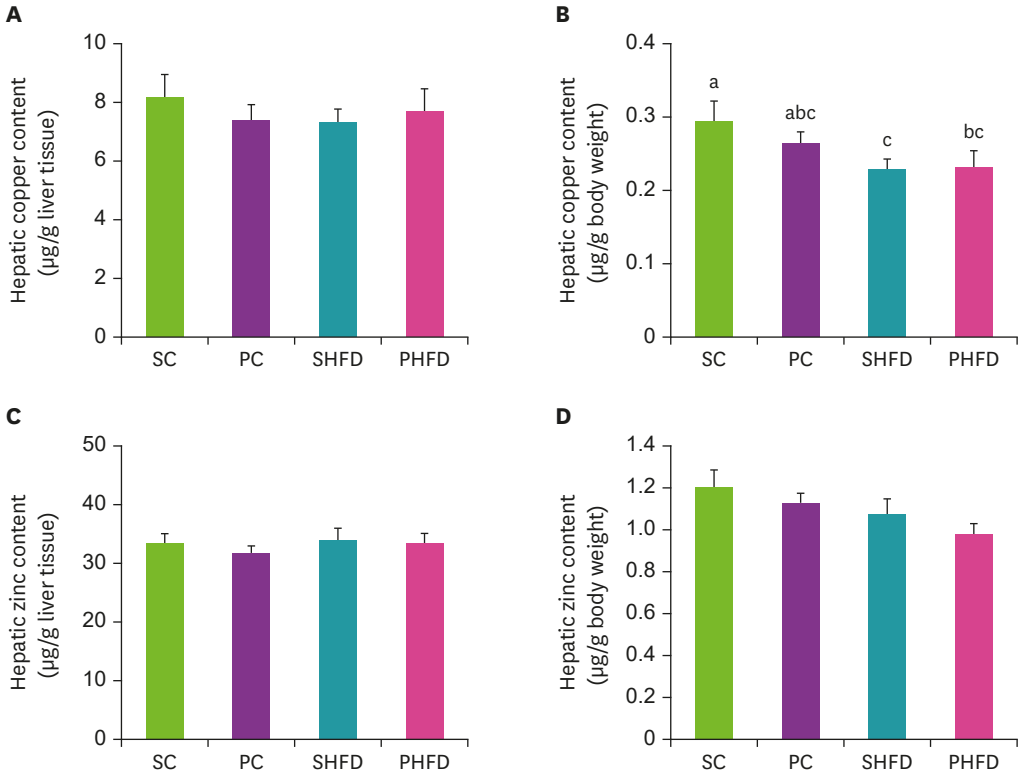

Fig. 2. Hepatic copper content (A), hepatic copper adjusted by body weight (B), hepatic zinc content (C), and hepatic zinc adjusted by body weight (D). Values are expressed as mean ± SEM (n = 10–11 per group). Two-way analysis of variance followed by LSD post-hoc test was performed, and different letters indicate statistical significance ($p < 0.05$).
SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil

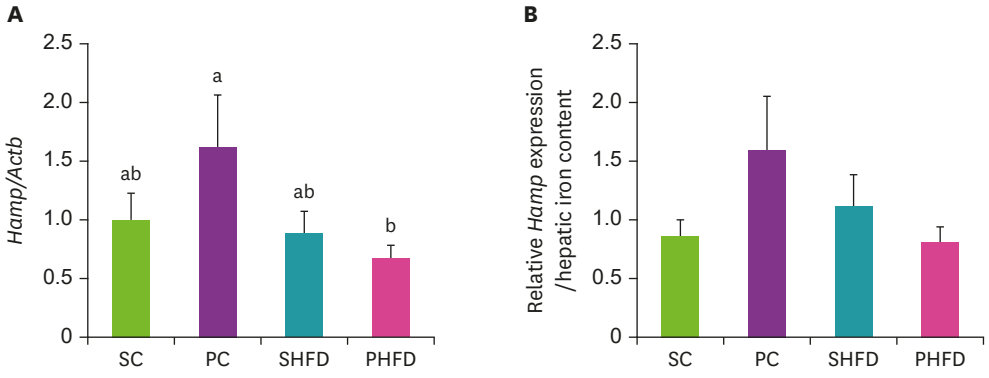

Fig. 3. Hepatic *Hamp* mRNA expression (A) and Hepatic *Hamp* mRNA expression adjusted by hepatic iron content (B). Hepatic *Hamp* mRNA expression was normalized relative to hepatic *Actb* mRNA expression (A), and the relative *Hamp* expression is adjusted by hepatic iron content (μg/g liver tissue) (B). Values are expressed as mean ± SEM (n = 6 per group). Two-way analysis of variance followed by LSD post-hoc test was performed, and different letters indicate statistical significance ($p < 0.05$). *Hamp* mRNA expression data, which was not normally distributed, was transformed before conducting two-way ANOVA, but the graph presents the original data.
Hamp, hepcidin; *Actb*, β-actin; SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil.

DMT1), *Slc11a2* (a divalent metal transporter DMT1 encoding gene), *Slc40a1* (an iron exporter Ferroportin encoding gene), and *Heph* (an intestinal ferroxidase Hephaestin encoding gene) were measured.

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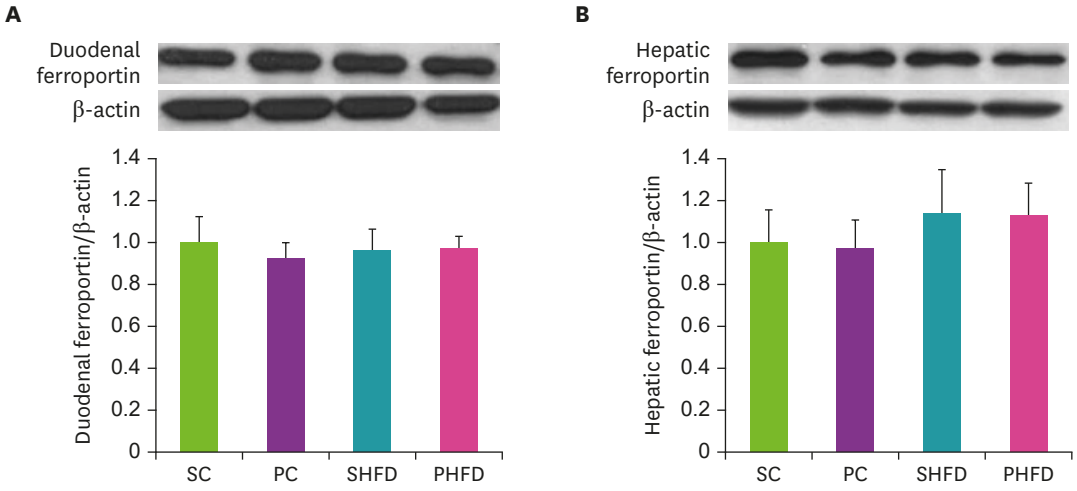

Fig. 4. Ferroportin protein expression in duodenum (A) and the liver (B). Representative images are presented. Ferroportin protein expression levels are quantified and normalized relative to β-actin protein level. The relative values are expressed as mean ± SEM (n = 6 per group). Two-way analysis of variance was performed. SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil.

HFD-fed mice had lower *Dcytb* mRNA expression compared to the control diet-fed mice ($p = 0.037$), and the SHFD group had significantly lower expression of *Dcytb* compared to the SC group ($p = 0.018$). Compared to SBO-fed mice, PNO-fed mice had significantly lower *Slc11a2* ($p = 0.022$) and *Slc40a1* expression ($p = 0.027$), and the PC group had significantly lower *Slc11a2* expression than the SC group ($p = 0.034$). Although there were no overall effects of the fat amount or the oil type on the *Heph* expression level, a significant interaction between the two factors was observed ($p = 0.037$). The PC group had significantly higher *Heph* expression than SC group ($p = 0.023$), but there was no difference between SHFD and PHFD groups (**Fig. 5**).

DISCUSSION

In this study, HFD feeding lowered hepatic iron storage regardless of the oil type. SHFD and PHFD groups had lower hepatic iron content compared to SC and PC, respectively. The lower hepatic iron status induced by HFD has been reported by previous studies [4-6]. Since mice in this study were fed less amount of fat (45% vs. 60% kcal fat) for shorter duration (12 vs. 16 weeks) compared to the earlier studies [4,5], the magnitude of hepatic iron reduction was smaller (25% vs. 70% reduction). This suggests a possibility that hepatic iron storage is controlled by adiposity, and the negative correlation between hepatic iron content and body weight or WAT mass observed in the current study supports this. Based on our calculation, 1 gram of weight gain induces 4 percent decrease in hepatic iron content.

The partial replacement of SBO with PNO in the HFD led to 18% less weight gain (4 g difference) and 18% less white fat mass (0.96 g difference), but it did not restore the reduced hepatic iron storage induced by HFD feeding. When mice were fed 60% kcal fat diet for 15 weeks and switched to 10% kcal fat diet and fed for 16 weeks, their body weight was 35% lower (23.7 g difference) and hepatic iron content was 30% higher (17 μg/g tissue difference) compared to the mice fed 60% kcal fat diet for 31 weeks [6]. This suggests that the weight difference between SHFD and PHFD groups is not sufficient to improve hepatic iron status.

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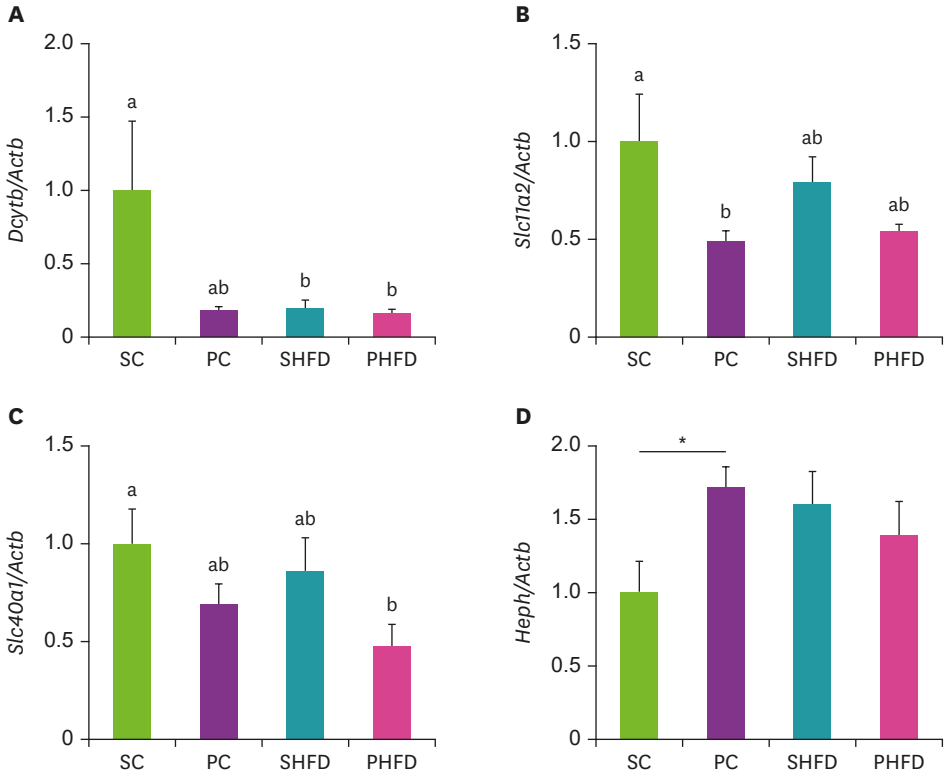

Fig. 5. Duodenal mRNA expression of *Dcytb* (A), *Slc11a2* (B), *Slc40a1* (C) and *Heph* (D). Duodenal gene expression was normalized relative to duodenal *Actb* mRNA expression. Values are expressed as mean ± SEM (n = 6 per group). Two-way ANOVA followed by LSD post-hoc test was performed, and different letters indicate statistical significance ($p < 0.05$). *Dcytb* and *Slc11a2* mRNA expression data, which were not normally distributed, were transformed before conducting two-way ANOVA, but the graphs present the original data. Due to the interaction between fat amount and oil type of the diets for *Heph* expression, Student's t-test was used to determine statistical significance, and asterisks indicate statistical significance ($p < 0.05$).
Dcytb, duodenal cytochrome b reductase; *Actb*, β-actin; *Slc11a2*, divalent metal transporter 1; *Slc40a1*, ferroportin; *Heph*, hephaestin; SC group, control diet containing 10% kcal from SBO; PC group, control diet containing 10% kcal from PNO; SHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from SBO; PHFD group, high-fat diet containing 35% kcal from lard and 10% kcal from PNO; PNO, Korean pine nut oil; SBO, soybean oil; ANOVA, analysis of variance.

To determine the mechanism by which HFD feeding decreased hepatic iron storage, we measured hepatic *Hamp* mRNA expression, and hepatic and duodenal Ferroportin protein expression levels. Hepcidin, a key regulator of iron homeostasis, controls plasma iron concentration and tissue distribution of iron through post-translational regulation of Ferroportin, an iron exporter [18]. When plasma iron concentration is high, Hepcidin binds to Ferroportin, inducing internalization, ubiquitination, and degradation of Ferroportin, and inhibits iron release from enterocytes, hepatocytes, splenocytes, and macrophages into the circulation [18]. Obesity is reported to upregulate *Hamp* expression through release of pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-α [19], and this can lead to iron deficiency [20].

However, in this study, HFD-fed mice were shown to have lower hepatic *Hamp* expression than control-diet fed mice. Similar results have been previously reported, showing that severely obese mice with hepatic iron depletion had lower *Hamp* expression levels than lean mice [4,5]. This indicates that the severe hepatic iron depletion may downregulate *Hamp* expression to overcome the depletion, and this effect is greater than the upregulation of *Hamp* expression induced by obesity. Although the hepatic *Hamp* expression level adjusted by hepatic iron content was significantly higher in severe obese mice compared to lean mice [4], the value of the current study was not different between control diet- and HFD-fed mice. This

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may be because the magnitude of hepatic iron reduction in the HFD groups was not as great as those observed in the previous studies [4,5]. The expression of Ferroportin, regulated by Hepcidin, was not different among the groups.

Slc11a2 encodes DMT1, which transports Fe^{2+} from the gut lumen to enterocytes, and the reduced hepatic iron storage in HFD-fed mice did not alter duodenal *Slc11a2* mRNA levels in this study. This is inconsistent with the previous report [21] showing that HFD group had significantly lower plasma iron concentration and higher *Slc11a2* mRNA levels than control group. The lower level of plasma iron concentration indicates severe iron depletion, and iron supplementation to HFD-fed mice was shown to reverse the HFD-induced upregulation of *Slc11a2* expression [21]. Therefore, the upregulation of *Slc11a2* mRNA in the HFD group could be a compensatory mechanism to alleviate the reduction of iron storage. As mentioned before, the small reduction of hepatic iron storage in this study seems to be insufficient to upregulate *Slc11a2* expression.

For non-heme iron to be absorbed via DMT1, Fe^{3+} should be reduced to Fe^{2+} by duonenal cytochrome b reductase (encoded by *Dcytb* gene). In this study, HFD-fed mice had lower *Dcytb* mRNA expression compared to control diet-fed mice, and the SHFD group had significantly lower expression of *Dcytb* than the SC group, indicating that the Fe^{3+} was not effectively reduced and absorbed in HFD-induced obesity. HFD feeding has been reported to downregulate *Dcytb* mRNA expression even when iron was enriched to diets [21]. In *Dcytb* knockout mice, which had lower iron uptake into enterocytes, iron release to the blood was promoted by upregulation of Ferroportin (encoded by *Slc40a1*) and Hephaestin (encoded by *Heph*) expression as a compensatory mechanism [22]. Hephaestin, oxidizing Fe^{2+} to Fe^{3+}, is essential for basolateral transfer because apo-transferrin, which transports iron in the blood, accepts Fe^{3+} only [8,9]; therefore, *Sla* mice, in which Hephaestin is mislocalized, have iron transport defect [23]. However, HFD feeding did not induce upregulation of duodenal *Slc40a1* and *Heph* mRNA levels in this study, and this may have led to the reduction of hepatic iron storage in HFD mice.

On the other hand, PNO-fed mice had significantly lower *Slc11a2* and *Slc40a1* expression compared to SBO-fed mice. Also, *Dcytb* expression of the PC group seemed to be as lower as that of HFD-fed mice. These indicate that PNO may interfere intestinal iron absorption. However, PNO did not induce hepatic iron depletion (no difference in hepatic iron storage between SBO- and PNO-fed mice), suggesting that possible compensatory mechanisms exist. The upregulation of *Heph* expression in the PC group compared to the SC group is considered as one of the mechanisms, and it may help to overcome the lower level of *Dcytb* mRNA expression and maintain hepatic iron status of the PC group.

Hepatic copper and zinc content were not altered by the fat amount or the oil type although the expression of *Slc11a2* (DMT1 encoding gene), by which copper and zinc are partially absorbed, were significantly lower in PNO-fed mice. In Caco-2 enterocytes, knockdown of *Slc11a2* using antisense oligonucleotide treatment [24] or shRNA transfection [25] was shown to inhibit both iron and copper uptake [24] or to lower intracellular zinc content [25]. No difference in hepatic copper and zinc contents in the current study suggests that HFD-induced obesity may not significantly influence other absorption mechanisms of copper and zinc. Cu^{+} can be transported through copper transporter 1 (CTR1), and zinc can be transported through zinc transporter 4 (ZIP4) into enterocytes [26]. However, we cannot rule out the potential effects of HFD on the copper homeostasis because the hepatic

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copper content adjusted by body weight was significantly lower in HFD-fed mice compared to control diet-fed mice. This indicates that severe obesity could suppress intestinal copper absorption and reduce hepatic copper storage. The HFD-induced lower mRNA expression of *Dcytb* in this study can also support the possibility. Cu^{2+} should be reduced to Cu^{+} by duodenal cytochrome b reductase to be absorbed through CTR1 [27]; therefore, when *Dcytb* is not expressed enough, dietary copper is absorbed only by DMT1 competing with other divalent cations, which decreases the efficiency of copper absorption.

A limitation of this study is that expression of hepatic and duodenal genes related to iron absorption, except of Ferroportin, was determined at the mRNA level only. Nevertheless, it is considered that the gene expression at the mRNA levels can be utilized to understand the effects of PNO since the mRNA levels of the genes were reported to be closely related to the phenotype [6,28,29]. Additional analyses, including Western blot, will be helpful to confirm the PNO effect on hepatic and intestinal iron metabolism.

SUMMARY

HFD feeding reduced hepatic iron content, and lowered hepatic *Hamp* and duodenal *Dcytb* mRNA expression. Substitution of SBO with PNO in HFD resulted in the less weight gain and less white fat mass, but it was not enough to restore iron depletion caused by HFD-induced obesity. Compared to SBO-fed mice, PNO-fed mice had significantly lower *Slc11a2* and *Slc40a1* expression, but it did not induce hepatic iron depletion in PNO-fed mice. Hepatic copper and zinc content were not lower in HFD-fed mice, indicating that hepatic copper and zinc status may be less sensitive to body adiposity compared to hepatic iron status since there are additional copper and zinc absorption mechanisms.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

The fatty acid composition of the experimental diets (% of lipid)

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