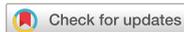


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Dietary supplementation with Korean pine nut oil decreases body fat accumulation and dysregulation of the appetite-suppressing pathway in the hypothalamus of high-fat diet-induced obese mice

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ABSTRACT

BACKGROUND/OBJECTIVES: Korean pine nut oil (PNO) has been reported to suppress appetite by increasing satiety hormone release. However, previous studies have rendered inconsistent results and there is lack of information on whether dietary Korean PNO affects the expression of satiety hormone receptors and hypothalamic neuropeptides. Therefore, our study sought to evaluate the chronic effects of Korean PNO on the long-term regulation of energy balance.

MATERIALS/METHODS: Five-week-old male C57BL/6 mice were fed with control diets containing 10% kcal fat from Korean PNO or soybean oil (SBO) (PC or SC) or high-fat diets (HFDs) containing 35% kcal fat from lard and 10% kcal fat from Korean PNO or SBO (PHFD or SHFD) for 12 weeks. The expression of gastrointestinal satiety hormone receptors, hypothalamic neuropeptides, and genes related to intestinal lipid absorption and adipose lipid metabolism was then measured.

RESULTS: There was no difference in the daily food intake between PNO- and SBO-fed mice; however, the PC and PHFD groups accumulated 30% and 18% less fat compared to SC and SHFD, respectively. Korean PNO-fed mice exhibited higher messenger RNA (mRNA) expression of *Ghsr* (ghrelin receptor) and *AgRP* (agouti-related peptide) ($P < 0.05$), which are expressed when energy consumption is low to induce appetite as well as the appetite-suppressing neuropeptides *Pomc* and *Cartpt* ($P = 0.079$ and 0.056 , respectively). Korean PNO downregulated jejunal *Cd36* and epididymal *Lpl* mRNA expressions, which could suppress intestinal fatty acid absorption and fat storage in white adipose tissue. Consistent with these findings, Korean PNO-fed mice had higher levels of fecal non-esterified fatty acid excretion. Korean PNO also tended to downregulate jejunal *Apoa4* and upregulate epididymal *Adrb3* mRNA levels, suggesting that PNO may decrease chylomicron synthesis and induce lipolysis.

CONCLUSIONS: In summary, Korean PNO attenuated body fat accumulation, and appeared to prevent HFD-induced dysregulation of the hypothalamic appetite-suppressing pathway.

Keywords: Neuropeptides; gastrointestinal hormones; intestinal absorption; adipose tissue; lipid metabolism

Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Han SN; Funding acquisition: Han SN; Investigation: Shin S, Park S, Lim Y; Supervision: Han SN; Writing - original draft: Shin S, Han SN; Writing - review & editing: Shin S, Han SN.

INTRODUCTION

Gastrointestinal organs and associated visceral organs, such as the pancreas, liver, and adipose tissue control energy balance (i.e., the balance between energy intake and energy expenditure) by regulating satiety or hunger signals. They communicate with the hypothalamus, a key appetite control center, through neural and endocrine pathways [1]. Neurons in the hypothalamus express appetite-stimulating or suppressing neuropeptides in response to signals from the periphery to maintain energy homeostasis. Dysregulation of these systems results in chronic positive energy balance, thus leading to obesity [2] and the development of chronic diseases such as dyslipidemia, hypertension, type 2 diabetes, and cardiovascular diseases [3]. Therefore, dietary bioactive compounds capable of improving the regulation of energy homeostasis could be used as effective and safe nutraceutical strategies to prevent obesity and obesity-related chronic diseases.

Pine nuts have been consumed for centuries around the world, especially in the Mediterranean and Asia, and approximately 60% of the weight of pine nuts is fat. The fatty acid composition of Korean pine nut (*Pinus Koraiensis*) oil (PNO) is 4% palmitic acid (16:0), 28% oleic acid (18:1, Δ9), 47% linoleic acid (18:2, Δ9,12), and 14% pinolenic acid (18:3, Δ5,9,12) [4]. Pinolenic acid, a unique fatty acid found in PNO, is an unsaturated polymethylene-interrupted fatty acid with a cis-5 ethylenic bond (Δ5-UPIFA) [5] and a positional isomer of γ-linolenic acid [6]. Korean PNO contains a much higher fraction of pinolenic acid than other PNOs including Italian stone PNO [7].

Many studies have confirmed the health benefits of Korean PNO. In a mouse model of diet-induced obesity, Korean PNO suppressed intestinal lipid uptake [8], increased fatty acid oxidation in skeletal muscle [9], and induced thermogenesis in brown adipose tissue (BAT) [9]. Korean PNO was also shown to attenuate high-fat diet (HFD)-induced hepatic lipid accumulation in mice [10], and blood pressure in spontaneously hypertensive rats [11]. In clinical studies, Korean PNO increased the release of satiety hormones and decreased appetite in overweight post-menopausal women [7] and decreased dietary intake in overweight women [12]. However, another study reported no effect of Korean PNO on appetite and energy intake [13]. Moreover, no previous studies have evaluated the chronic effects of Korean PNO on the expression of satiety hormone receptors and neuropeptides, which play a significant role in food intake, energy expenditure, and weight control [2]. Therefore, our study sought to examine the effects of Korean PNO on the long-term regulation of energy balance, including satiety hormones, neuropeptides, dietary lipid absorption, and adiposity, in mice fed with a HFD (45% kcal fat) or control diet (10% kcal fat) for 12 weeks.

MATERIALS AND METHODS

Animals and diets

Five-week-old male C57BL/6N mice were purchased from Central Laboratory Animal Inc. (Seoul, Korea) and maintained on a chow diet for 3 days before being assigned to 1 of 4 experimental diets: control diets containing 10% kcal fat from Korean PNO or soybean oil (SBO) (PC or SC) or HFDs containing 35% kcal fat from lard and 10% kcal fat from Korean PNO or SBO (PHFD or SHFD). The mice (n = 10-11 per group) were fed with the experimental diets *ad libitum* for 12 weeks. **Table 1** shows the composition of the experimental diets. Korean PNO, generously provided by Dubio Co., Ltd. (Hwaseong, Korea), was sent to

Table 1. Composition of the experimental diet¹⁾

Ingredient	Control diets (10% kcal fat)		High-fat diets (45% kcal fat)	
	g	kcal	g	kcal
Casein	200	800	200	800
L-cystine	3	12	3	12
Sucrose	350	1,400	172.8	691
Cornstarch	315	1,260	72.8	291
Dyetrose	35	140	100	400
Korean PNO or SBO	45	405	45	405
Lard	0	0	157.5	1,417.5
t-Butylhydroquinone	0.009	0	0.009	0
Cellulose	50	0	50	0
Mineral mix ²⁾	35	0	35	0
Vitamin mix ³⁾	10	40	10	40
Choline bitartrate	2	0	2	0
Total	1,045.0	4,057.0	848.1	4,056.5
kcal/g diet	3.85		4.73	

PNO, pine nut oil; SBO, soybean oil.

¹⁾Resource: Dyets, Inc., Bethlehem, PA, USA.

²⁾Thirty-five g of mineral mix (Research Diets, Inc., New Brunswick, 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 g of vitamin mix (Research Diets, Inc., V10001) provides 4000 IU vitamin A, 1000 IU vitamin D3, 50 IU vitamin E, 30 mg niacin, 16 mg pantothenic acid, 7 mg vitamin B6, 6 mg vitamin B1, 6 mg vitamin B2, 2 mg folic acid, 0.5 mg menadione, 0.2 mg biotin, 10 µg vitamin B12 and 9.78 g sucrose.

Dyets Inc. (Bethlehem, PA, USA) to prepare the experimental diets. All mice were housed individually under controlled temperature ($23 \pm 3^\circ\text{C}$) and humidity ($55 \pm 10\%$) conditions with a 12 hour-dark/light cycle. Body-weight was measured once a week, and food intake was measured 4 times a week. At the end of the experimental period, the mice were euthanized in a CO₂ chamber after a 12-h fast. Brain, stomach, small intestine, and white adipose tissues (WAT) were dissected. All tissues were cleaned, flash-frozen in liquid nitrogen, and stored at -80°C until required for analyses. All animal procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Seoul National University (approval No. SNU-101029-1).

Determination of fecal triacylglycerol (TAG), non-esterified fatty acids (NEFAs), and cholesterol concentrations

Feces were collected for 3 days during the 12th week of the feeding experiment, and fecal TAG, NEFA, and cholesterol concentrations were determined via an enzymatic assay using a commercial kit (Asan Pharmacy, Seoul, Korea) following the manufacturer's instructions.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from the hypothalamus and the remaining brain tissue, as well as from the stomach, jejunum, and epididymal WAT using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 µg of total RNA using the PrimeScript II 1st strand cDNA synthesis kit (Takara, Kusatsu, Japan).

Quantification of gene expression

To determine the effects of PNO on the appetite control pathway, the mRNA levels of cholecystokinin related genes (jejunal *Cck*, and cerebral *Cckar* and *Cckbr*), ghrelin related genes (gastric *Ghrl* and *Goat*, and hypothalamic *Ghsr*), and neuropeptides (hypothalamic *Npy*, *AgRP*, *Pomc*, and *Cartpt*) were quantified. To examine the influence of PNO on lipid metabolism, the mRNA levels of genes associated with lipid absorption and metabolism in the small

Table 2. The primer sequences used for real-time polymerase chain reaction

Gene	Forward primer	Reverse primer
<i>Cck</i>	TCC AGC AGG TCC GCA AA	CCA GGC TCT GCA GGT TCT TAA
<i>Cckar</i>	ATA AAA GTT GGA GTA TTG TGT GAG CTT C	TTA AGT GTT TTC AAC ACT AAT TTT GCA
<i>Cckbr</i>	TGG GAC CTA ACC CTA CTC CGG TGA T	CAA ATG AGA GGG TGT ACT CAG GA
<i>Ghrl</i>	TCC AAG AAG CCA CCA GCT AA	AAC ATC GAA GGG AGC ATT GA
<i>Goat</i>	ATT TGT GAA GGG AAG GTG GAG	CAG GAG AGC AGG GAA AAA GAG
<i>Ghsr</i>	ACC GTG ATG GTA TGG GTG TCG	CAC AGT GAG GCA GAA GAC CG
<i>Npy</i>	TCC GCT CTG CGA CAC TAC AT	TGC TTT CCT TCA TTA AGA GGT CTG
<i>AgRP</i>	AGC TTT GGC GGA GGT GCT	GCC ACG CGC AGA ACG A
<i>Pomc</i>	TGA ACA TCT TTG TCC CCA GAG A	TGC AGA GGC AAA CAA GAT TGG
<i>Cartpt</i>	GCC AAG GCG GCA ACT C	TCT TGC AAC GCT TCG ATC TG
<i>Cd36</i>	CCA AGC TAT TGC GAC ATG ATT	TCT CAA TGT CCG AGA CTT TTC A
<i>Fabp2</i>	AGA GGA AGC TTG GAG CTC ATG ACA	TCG CTT GGC CTC AAC TCC TTC ATA
<i>Dgat2</i>	TGG GTC CAG AAG AAG TTC CAG AAG TA	ACC TCA GTC TCT GGA AGG CCA AAT
<i>Apoa4</i>	TTC CTG AAG GCT GCG GTG CTG	CTG CTG AGT GAC ATC CGT CTT CTG
<i>Lpl</i>	TTA TCC CAA TGG AGG CAC TTT C	CAC GTC TCC GAG TCC TCT CTC T
<i>Plin1</i>	CAT CTC TAC CCG CCT TCG AA	TGC TTG CAA TGG GCA CAC TG
<i>Ucp2</i>	CAG GTC ACT GTG CCC TTA CCA	CAC TAC GTT CCA GGA TCC CAA
<i>Adrb3</i>	ACC AAC GTG TTC GTG ACT	ACA GCT AGG TAG CGG TCC
<i>Pparg</i>	CAG CAG GTT GTC TTG GAT GTC	AGC CCT TTG GTG ACT TTA TGG
<i>Ppargc1a</i>	CCG TAA ATC TGC GGG ATG ATG	CAG TTT CGT TCG ACC TGC GTA A
<i>Ppard</i>	AGC CAT ATT CCC AGG CTG TCT C	CCT AGG CAG CAC AAG GGT CAT
<i>Gapdh</i>	GGA GAA ACC TGC CAA GTA	AAG AGT GGG AGT TGC TGT TG

Cck, cholecystokinin; *Cckar*, cholecystokinin A receptor; *Cckbr*, cholecystokinin B receptor; *Ghrl*, ghrelin; *Goat*, ghrelin O-acyltransferase; *Ghsr*, growth hormone secretagogue receptor; *Npy*, neuropeptide Y; *AgRP*, agouti-related peptide; *Pomc*, pro-opiomelanocortin- α ; *Cartpt*, cocaine- and amphetamine-regulated transcript prepeptide; *Cd36*, fatty acid translocase; *Fabp2*, fatty acid binding protein 2, intestinal; *Dgat2*, diacylglycerol O-acyltransferase 2; *Apoa4*, apolipoprotein A-IV; *Lpl*, lipoprotein lipase; *Plin1*, perilipin 1; *Ucp2*, mitochondrial uncoupling protein 2; *Adrb3*, beta-3 adrenergic receptor; *Pparg*, peroxisome proliferator-activated receptor gamma; *Ppargc1a*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *Ppard*, peroxisome proliferator-activated receptor delta; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

intestine (jejunal *Cd36*, *Fabp2*, *Dgat2*, and *Apoa4*) and those in WAT (epididymal *Lpl*, *Plin1*, *Ucp2*, *Adrb3*, *Pparg*, *Ppargc1a*, and *Ppard*) were quantified via real-time polymerase chain reaction (PCR) using SYBR Premix Ex Taq (Takara) and a StepOne Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicate. The results were analyzed with the StepOne™ software version 2.1 (Applied Biosystems) and all values were normalized to the levels of the house-keeping gene *Gapdh*. **Table 2** summarizes the specific primer sequences used to conduct the aforementioned experiments.

Statistical analysis

All statistical analyses were conducted using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance was conducted to identify the overall effects of dietary fat amount, oil type, and the interaction between these 2 factors, followed by Fisher's LSD multiple comparison test for individual group comparisons. Pearson's correlation was used to determine the linear relationship between variables. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Body weight change, food intake, feed efficiency, and WAT weight

HFD-fed mice exhibited significantly higher weight gain ($P < 0.001$) and WAT amount ($P < 0.001$) than the mice fed with the control diet. Mice fed with diets containing Korean PNO gained less weight gain ($P = 0.014$) and exhibited less white fat mass ($P = 0.004$) than

mice fed with the SBO-containing diets. There were no significant differences in daily food intake and daily energy intake between the mice fed with the diets containing SBO or PNO; however, feed efficiency, a ratio of weight gain to food intake, was lower ($P = 0.007$) in the mice fed with the PNO-containing diets (Table 3).

The PHFD group exhibited significantly lower body weight (10% less, $P = 0.022$), less weight gain (18% less, $P = 0.015$), and lower feed efficiency (17% less, $P = 0.012$) than the SHFD group. The PC and PHFD groups had significantly less WAT compared to the SC and SHFD groups (30% less, $P = 0.046$; 18% less, $P = 0.033$; respectively). Some of the results in Table 3 have been reported in our previous studies (10, 14), in which the effects of Korean PNO on immune response and hepatic triglyceride lipid accumulation were investigated using the same animals.

Fecal lipid concentrations

HFD-fed mice had higher fecal TAG (6.8 times higher, $P < 0.001$) and NEFA excretion (3.2 times higher, $P < 0.001$) than control diet-fed mice, whereas cholesterol levels did not differ. Further, fecal NEFA levels were significantly higher in PHFD-fed mice than SHFD-fed mice (58% higher, $P = 0.044$; Fig. 1).

Table 3. Body weight, weight gain, food intake, feed efficiency, white adipose tissue weight, and serum leptin level

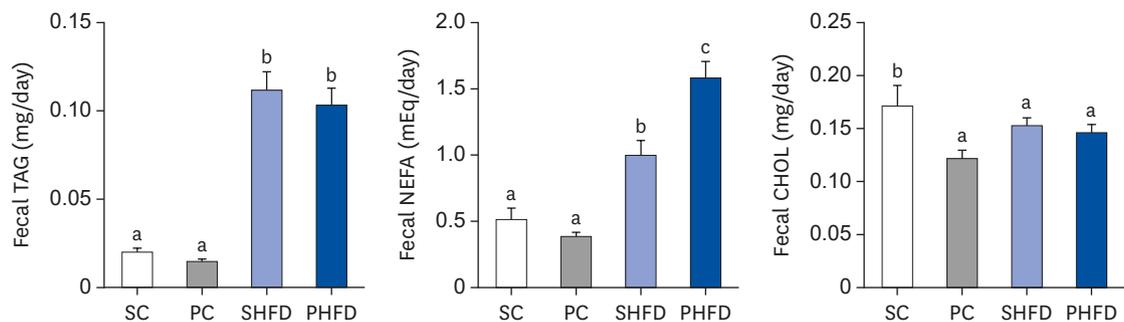
Parameter	Control diets		High-fat diets		Fat amount (P-value)	Oil type (P-value)	Interaction (P-value)
	SC	PC	SHFD	PHFD			
Body weight at 0 wk (g)	17.30 ± 0.51	16.74 ± 0.45	17.01 ± 0.36	17.04 ± 0.34	0.973	0.555	0.497
Body weight at 12 wk (g)	32.76 ± 0.96 ^{ab}	30.51 ± 0.64 ^a	38.49 ± 1.45 ^c	34.58 ± 1.42 ^b	< 0.001	0.012	0.487
Body weight gain (g)	15.48 ± 0.84 ^{ab}	13.76 ± 0.59 ^a	21.48 ± 1.42 ^c	17.53 ± 1.31 ^b	< 0.001	0.014	0.321
Daily food intake (g)	3.20 ± 0.06 ^b	3.20 ± 0.31 ^b	2.82 ± 0.05 ^a	2.76 ± 0.04 ^a	< 0.001	0.539	0.476
Daily energy intake (kcal)	11.80 ± 0.21 ^a	11.82 ± 0.11 ^a	13.11 ± 0.22 ^b	12.82 ± 0.20 ^b	< 0.001	0.495	0.431
Feed efficiency (mg/kcal) ¹⁾	15.56 ± 0.68 ^a	13.83 ± 0.52 ^a	19.43 ± 1.11 ^b	16.18 ± 1.03 ^a	0.001	0.007	0.395
White adipose tissue (g) ²⁾	3.10 ± 0.22 ^b	2.18 ± 0.18 ^a	5.34 ± 0.38 ^c	4.38 ± 0.39 ^d	< 0.001	0.004	0.947

Data are presented as means ± SEM, n = 10–11 for each group. Different superscripts indicate significant differences, $P < 0.05$.

SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; WAT, white adipose tissue.

¹⁾Feed efficiency (mg/kcal) = weight gain (mg)/total food intake (kcal).

²⁾WAT includes inguinal subcutaneous, epididymal, perirenal, and retroperitoneal WAT.



Fat amount (P-value)	< 0.001	< 0.001	0.837
Oil type (P-value)	0.409	0.044	0.056
Interaction (P-value)	0.843	0.002	0.139

Fig. 1. Fecal triacylglycerol, non-esterified fatty acid, and cholesterol levels. Data are presented as means ± SEM, n = 7–14 for each group. Two-way analysis of variance was used to determine the significant effect of fat amount and oil type. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test.

SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; TAG, triacylglycerol; NEFA, non-esterified fatty acid; CHOL, cholesterol.

Expression of genes involved in appetite control

The expression of hypothalamic *Ghsr*, a ghrelin receptor, was significantly affected by both fat amount and oil type ($P = 0.028$, $P = 0.027$, respectively). The *Ghsr* mRNA level of the PC group was 1.23-fold higher ($P = 0.024$) than that of the SC group. *Ghsr* mRNA levels showed negative correlation with body weight ($r = -0.467$, $P = 0.025$) and white fat mass ($r = -0.460$, $P = 0.027$) at 12 week. However, the mRNA levels of *Cck* (a major satiating hormone) and its receptors *Cckar* and *Cckbr* in the brain, gastric *Ghrl*, (an appetite inducer), and gastric *Goat* (ghrelin O-acyltransferase; a ghrelin activating enzyme) were not influenced by fat amount or oil type (Table 4), and none of them showed significant correlation with body mass or fat mass (data not shown). The *Ghrl* mRNA level was not correlated with hypothalamic *Npy* or hypothalamic *Agrp* mRNA levels.

The mRNA level of *Agrp*, a neuropeptide that increases appetite and reduces energy expenditure, was significantly higher in mice fed with Korean PNO-containing diets ($P = 0.022$). The PHFD group had significantly higher *Agrp* expression (2.16-fold, $P = 0.023$) than the SHFD group. However, the mRNA level of *Npy*, another neuropeptide that acts similarly to *Agrp*, was not significantly influenced by fat amount or by oil type. The mRNA levels of hypothalamic *Pomc* and *Cartpt*, leptin-responsive neuropeptides that suppress appetite and increase energy expenditure, tended to be higher in mice fed with Korean PNO-containing diets (*Pomc*, $P = 0.079$; *Cartpt*, $P = 0.056$) (Fig. 2). Further, *Npy* and *Agrp* mRNA levels were negatively correlated with the weight (*Npy*, $r = -0.420$, $P = 0.046$; *Agrp*, $r = -0.465$, $P = 0.025$), whereas *Pomc* and *Cartpt* mRNA levels showed no correlation with the weight at 12 week (Fig. 3).

Expression of genes involved in lipid absorption

To investigate if the lower body mass, fat mass, and feed efficiency in Korean PNO-fed mice were caused by an alteration in intestinal fat absorption, the expression of genes involved in jejunal lipid metabolism was quantified (Fig. 4).

Overall, mice fed with diets containing Korean PNO had significantly lower mRNA levels of jejunal *Cd36*, which transports fatty acids from the gut lumen to enterocytes ($P = 0.025$). The PC group exhibited a lower yet non-significant *Cd36* mRNA expression trend (0.57-fold, $P = 0.085$) compared to the SC group. Mice fed with Korean PNO-containing diets also tended to have lower expression levels of jejunal *Apoa4*, which facilitates intestinal lipoprotein production ($P = 0.068$). Fat amount and oil type did not affect the expression of jejunal *Fabp2*, which transports and metabolizes fatty acids in enterocytes, and jejunal *Dgat2*, which synthesizes TAG from diacylglycerol (DAG).

Table 4. The mRNA expression levels of cholecystokinin- and ghrelin-related genes

Gene	Control diets		High-fat diets		Fat amount (<i>P</i> -value)	Oil type (<i>P</i> -value)	Interaction (<i>P</i> -value)
	SC	PC	SHFD	PHFD			
<i>Cck</i>	1.00 ± 0.26	0.99 ± 0.13	0.94 ± 0.15	1.04 ± 0.18	0.975	0.790	0.747
<i>Cckar</i>	1.00 ± 0.28	1.14 ± 0.64	0.43 ± 0.15	0.83 ± 0.32	0.292	0.510	0.744
<i>Cckbr</i>	1.00 ± 0.16	0.99 ± 0.17	0.93 ± 0.08	0.99 ± 0.19	0.901	0.858	0.748
<i>Ghrl</i>	1.00 ± 0.09	1.13 ± 0.15	1.02 ± 0.05	0.97 ± 0.18	0.600	0.745	0.511
<i>Goat</i>	1.00 ± 0.11	1.22 ± 0.08	1.08 ± 0.09	0.91 ± 0.14	0.286	0.812	0.086
<i>Ghsr</i>	1.00 ± 0.06 ^a	1.23 ± 0.09 ^b	0.92 ± 0.06 ^a	1.00 ± 0.05 ^a	0.028	0.027	0.265

Data are presented as means ± SEM, $n = 5-6$ for each group. All values are normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average expression level of SC group. Different superscripts indicate significant differences, $P < 0.05$.

mRNA, messenger RNA; SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; *Cck*, cholecystokinin; *Cckar*, cholecystokinin A receptor; *Cckbr*, cholecystokinin B receptor; *Ghrl*, ghrelin; *Goat*, ghrelin O-acyltransferase; *Ghsr*, growth hormone secretagogue receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

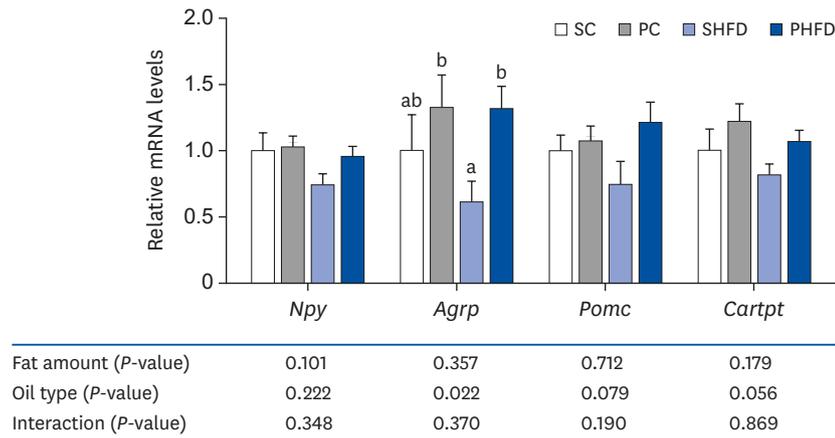


Fig. 2. The mRNA expression levels of neuropeptides (hypothalamic *Npy*, *Agrp*, *Pomc*, and *Cartpt*). Data are presented as means \pm SEM, $n=5-6$ for each group. Two-way analysis of variance was used to determine the significant effect of fat amount and oil type. Different letters indicate significant difference at $P < 0.05$ by Fisher's LSD multiple comparison test. All values are normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average expression level of SC group. mRNA, messenger RNA; SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; *Npy*, neuropeptide Y; *Agrp*, agouti-related peptide; *Pomc*, pro-opiomelanocortin-alpha; *Cartpt*, cocaine- and amphetamine-regulated transcript prepropeptide; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

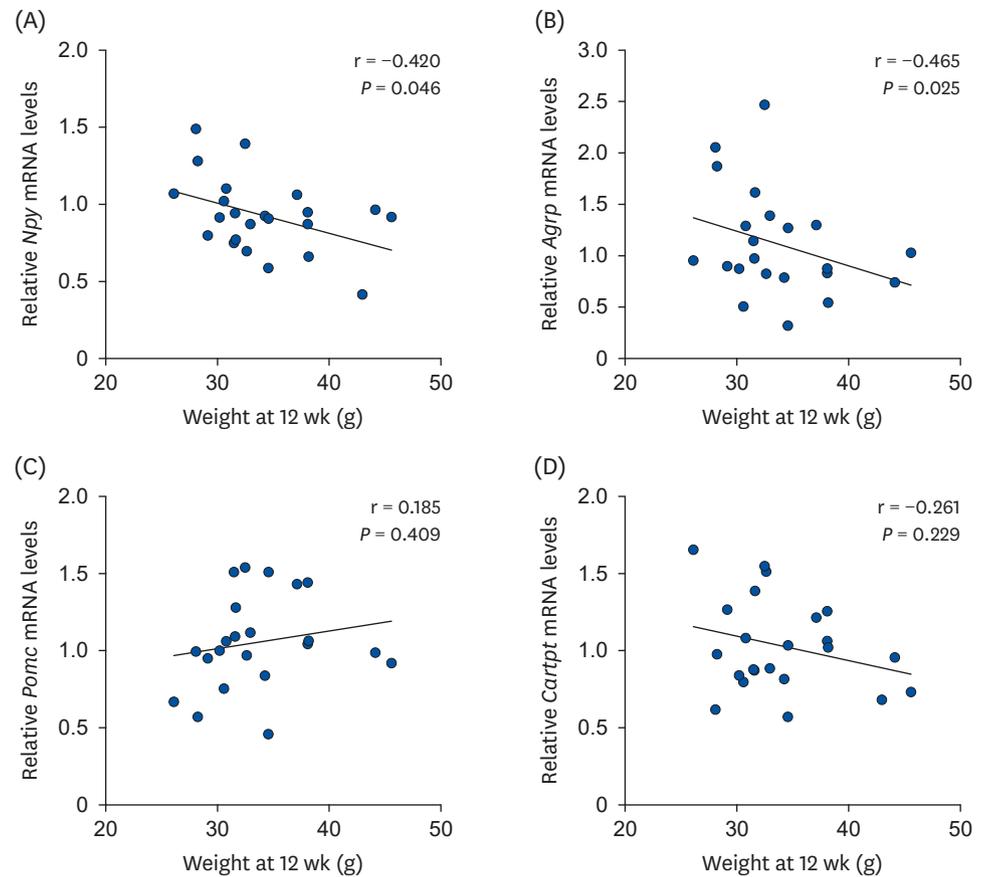
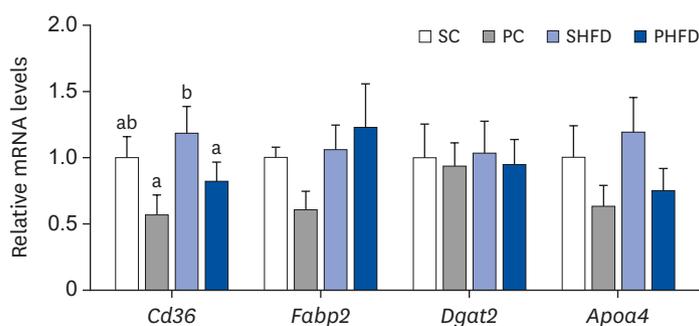


Fig. 3. Correlation between body weight and hypothalamic neuropeptide mRNA levels. Correlation between body weight and (A) *Npy*, (B) *Agrp*, (C) *Pomc*, and (D) *Cartpt* mRNA levels. Pearson's correlation was used to determine the linear relationship between variables. mRNA, messenger RNA; *Npy*, neuropeptide Y; *Agrp*, agouti-related peptide; *Pomc*, pro-opiomelanocortin-alpha; *Cartpt*, cocaine- and amphetamine-regulated transcript prepropeptide.



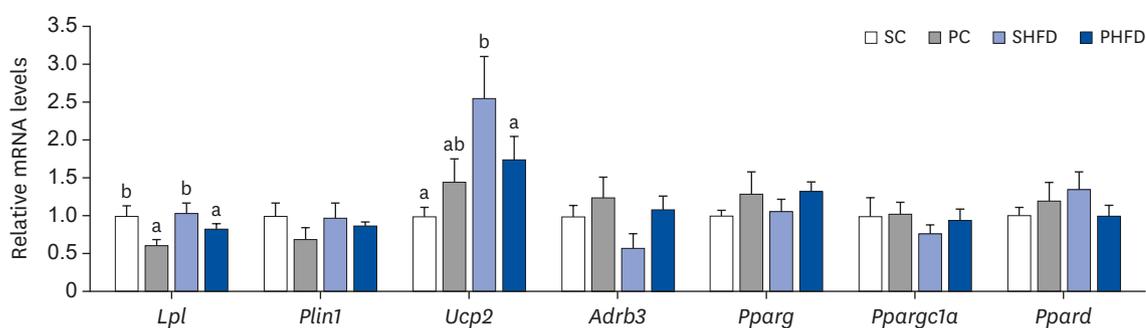
	Cd36	Fabp2	Dgat2	Apoa4
Fat amount (<i>P</i> -value)	0.204	0.132	0.922	0.469
Oil type (<i>P</i> -value)	0.025	0.602	0.738	0.068
Interaction (<i>P</i> -value)	0.851	0.207	0.964	0.878

Fig. 4. The mRNA expression levels of genes associated with intestinal lipid metabolism (jejunal *Cd36*, *Fabp2*, *Dgat2*, and *Apoa4*). Data are presented as means \pm SEM, $n = 5-6$ for each group. Two-way analysis of variance was used to determine the significant effect of fat amount and oil type. Different letters indicate significant difference, $P < 0.05$. All values are normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average expression level of SC group. mRNA, messenger RNA; SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; *Cd36*, fatty acid translocase; *Fabp2*, fatty acid binding protein 2, intestinal; *Dgat2*, diacylglycerol O-acyltransferase 2; *Apoa4*, apolipoprotein A-IV; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

Expression of genes involved in body fat accumulation

To examine whether Korean PNO reduced body fat mass by suppressing lipid storage or by enhancing lipid utilization, the expression of genes involved in lipid metabolism was measured in epididymal WAT (Fig. 5).

Mice fed with Korean PNO-containing diets had significantly lower expression levels of epididymal *Lpl* ($P = 0.020$), which hydrolyzes TAG within lipoproteins to DAG and fatty acids to facilitate fatty acid uptake by adipocytes. The *Lpl* mRNA level of the PC group was 38% lower than that of the SC group ($P = 0.035$). The mRNA level of epididymal *Adrb3*, which enhances lipolysis and thermogenesis in WAT, tended to be higher in mice fed with



	<i>Lpl</i>	<i>Plin1</i>	<i>Ucp2</i>	<i>Adrb3</i>	<i>Pparg</i>	<i>Ppargc1a</i>	<i>Ppard</i>
Fat amount (<i>P</i> -value)	0.306	0.643	0.021	0.164	0.814	0.345	0.716
Oil type (<i>P</i> -value)	0.020	0.205	0.643	0.081	0.160	0.514	0.736
Interaction (<i>P</i> -value)	0.461	0.513	0.101	0.536	0.953	0.668	0.187

Fig. 5. The mRNA expression levels of genes associated with lipid metabolism in the white adipose tissue (epididymal *Lpl*, *Plin1*, *Ucp2*, *Adrb3*, *Pparg*, *Ppargc1a*, and *Ppard*). Data are presented as means \pm SEM, $n = 5-6$ for each group. Different letters indicate significant difference, $P < 0.05$. All values are normalized to the levels of house-keeping gene *Gapdh* and expressed as relative mRNA level compared to the average expression level of SC group. mRNA, messenger RNA; SC, 10% soybean oil; PC, 10% pine nut oil; SHFD, 10% soybean oil + 35% lard; PHFD, 10% pine nut oil + 35% lard; *Lpl*, lipoprotein lipase; *Plin1*, perilipin 1; *Ucp2*, mitochondrial uncoupling protein 2; *Adrb3*, β_3 adrenergic receptor; *Pparg*, peroxisome proliferator-activated receptor gamma; *Ppargc1a*, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *Ppard*, peroxisome proliferator-activated receptor delta; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

Korean PNO containing diets ($P = 0.081$). The mRNA level of *Ucp2*, which plays a role in thermogenesis, was significantly higher in HFD-fed mice ($P = 0.021$). The mRNA levels of *Plin1*, *Pparg*, *Ppargc1a*, and *Ppard* were not affected by fat amount and oil type.

DISCUSSION

The HFD containing 10% kcal derived from Korean PNO fat and 35% from lard resulted in lower body mass increases and less white fat mass in mice compared to the HFD containing 10% kcal derived from SBO fat and 35% from lard even though the mice exhibited no differences in calorie consumption. Previously, mice fed with a HFD prepared with 30% kcal from Korean PNO fat and 15% from lard reportedly consumed less food (7.27% less g/day, 7.46% fewer calories/day) than those fed with a HFD made with 30% kcal from SBO fat and 15% from lard [14]; however, the substitution of 10% kcal Korean PNO fat for SBO in the control diet or the HFD did not induce any difference in food intake. This indicates that the substitution of 10% kcal fat with Korean PNO may not be enough to exert significant effects on appetite control, and the differences in body fat mass between SBO- or Korean PNO-fed mice may be caused by other characteristics of Korean PNO. Therefore, in this study, we investigated the chronic effects of dietary Korean PNO on molecular mechanisms of energy balance regulation, including the expression of satiety hormone receptors and neuropeptides.

We did not observe differences in the mRNA levels of *Cck*, a major satiety hormone, and its receptors *Cckar* and *Cckbr* among the experimental groups. This appeared to contradict a previous study that reported that Korean PNO promoted CCK release both in STC-1 enterocytes and in post-menopausal overweight women [7]. This discrepancy may have been caused by the 12-hour fasting period to which the mice were submitted prior to their euthanasia in this study. Given that CCK signals are induced when food is present in the gut lumen [15], fasting could have masked the satiating effect of PNO. However, it is also plausible that the expression of satiating peptides was not affected simply because the amounts of dietary Korean PNO were too low, which could also explain why no differences in food intake were observed between the SBO- and Korean PNO-containing diet groups.

The mRNA levels of *Ghrl*, an appetite-stimulating hormone, and *Goat*, a ghrelin activating enzyme, were not different among the experimental groups. This result was consistent with earlier studies that reported no difference in *Ghrl* and *Goat* expression between lean and diet-induced obese mice after fasting [16,17]. However, the mRNA level of ghrelin receptor *Ghsr* was significantly higher in the PC group than in other groups, and the *Ghsr* mRNA levels were negatively correlated with body weight and white fat mass (data not shown). *Ghsr* is known to increase appetite and decrease energy expenditure and is thus upregulated when more energy intake is needed [18]. Therefore, these data suggest that mice in the PC group, which had the lowest body fat amount among all groups, received a strong appetite-stimulating signal to promote energy consumption. Similarly, Korean PNO consumption was reported to mimic calorie restriction responses by reversing HFD-induced downregulation of SIRT3 (sirtuin 3; i.e., a protein that mediates calorie restriction) [10]. In contrast, *Ghrl* and *Goat* mRNA levels showed no correlation with body weight and fat mass (data not shown). This may indicate that the expression of gastrointestinal hormones and their associated enzymes is regulated by short-term satiation signals, whereas that of hormone receptors is regulated by long-term energy status signals.

Given that Korean PNO-fed mice had lower amounts of WAT, we speculated that they would also have lower expression levels of *Npy* and *Agrp* (neuropeptides that increase energy intake and reduce energy expenditure). However, contrary to our expectations, *Agrp* mRNA expression was significantly lower in the SHFD group compared to the PHFD group. A compensatory mechanism to decrease food intake and maintain energy balance was likely induced in the SHFD group mice, as they exhibited higher body weight and more WAT than the PHFD mice. This was further supported by the negative correlation of *Npy* and *Agrp* mRNA levels with body weight and white fat amount.

Pomc and *Cartpt* mRNA levels tended to be higher in Korean PNO-fed mice, whereas *Pomc* and *Cartpt* levels in the SHFD group were generally lower. Given that leptin stimulates *Pomc* and *Cartpt* expression [1], the SHFD group, which had the highest leptin level [10], was expected to have higher *Pomc* and *Cartpt* expression than the other groups. However, the SHFD group had lower *Pomc* and *Cartpt* mRNA levels, suggesting that leptin failed to induce an increase in *Pomc* and *Cartpt* expression. This, in turn, may have been caused by leptin resistance. Similar results were reported in previous animal studies. High-fat and high-sugar diet lowered *Pomc* mRNA levels in rats [19], and obese rats had lower *Pomc* expression than obesity-resistant rats [20]. In these 2 studies, *Pomc* downregulation induced hyperphagia and obesity [19,20]. Another study reported that HFD feeding for 8 weeks did not upregulate *Pomc* mRNA expression, and *Pomc* mRNA expression in HFD-fed mice was even lower than that of lean mice after 19 weeks. This dysregulation of the POMC/CART pathway was accompanied by severe leptin resistance, suggesting that leptin resistance was a possible cause [21]. Therefore, the lower fat mass of the PHFD group may have prevented the development of leptin resistance and POMC/CART pathway dysregulation.

Korean PNO upregulated fecal NEFA excretion, and fecal NEFA levels were significantly higher in mice fed with the Korean PNO-containing HFD than in mice fed with the SBO-containing HFD. Consistent with these observations, Korean PNO-fed mice exhibited lower *Cd36* expression and tended to have lower *Apoa4* expression in the jejunum. Dietary fatty acids in the gut lumen are transported by CD36 into enterocytes [22], and Apo A-IV facilitates the chylomicron packaging process [23]. Therefore, the lipids in Korean PNO-containing diets may not be efficiently absorbed and packaged into chylomicron particles compared to SBO-containing diets, which could have led to the lower fat mass and body mass of mice fed with the Korean PNO-containing diets. This was supported by one of our previous studies in which mice were fed with a HFD with 30% kcal fat from Korean PNO and 15% kcal fat from lard. The mice fed with the Korean PNO-containing diet tended to exhibit lower mRNA expression of *Mttp*, which is involved in TAG incorporation into chylomicrons [8].

Compared to SBO-fed mice, Korean PNO-fed mice had lower *Lpl* mRNA expression and tended to have higher *Adrb3* mRNA expression in epididymal WAT. These data suggest the potential benefits of Korean PNO for attenuating body fat accumulation by downregulating fatty acid uptake by adipocytes and upregulating lipolysis and thermogenesis in adipocytes. This is because LPL (lipoprotein lipase) in adipocytes cleaves fatty acids from TAG in chylomicrons [24], and activation of the β_3 -adrenergic receptor, encoded by the *Adrb3* gene, increases lipolysis and thermogenesis [25]. This is supported by another study in which substitution of 30% kcal fat with Korean PNO in a 45% kcal fat diet upregulated *Ucp1* expression in BAT at both the mRNA and protein levels [9]. Pinolenic acid, the key fatty acid in Korean PNO, upregulated thermogenic gene expression, including *Ucp1* and *Ppargc1a*, and augmented the norepinephrine-induced upregulation of the genes in mouse primary

adipocytes [26]. The higher *Ucp2* mRNA levels in the SHFD group were interpreted as a defense mechanism to maintain energy balance by increasing energy expenditure, which was supported by previous studies in which HFD upregulated *Ucp2* expression [27,28]. Although 45% kcal fat diets containing 30% kcal fat from Korean PNO upregulated *Ppargc1a* and *Ppard* mRNA expression in skeletal muscle [9], the expression of these genes in epididymal WAT was not different among the groups evaluated in the present study. This inconsistency may be due to the differences in Korean PNO content in the experimental diets or the differences in tissue characteristics. For instance, skeletal muscle is richer in mitochondria compared to other tissue types and could thus regulate fatty acid catabolism more sensitively.

In summary, dietary Korean PNO consumption reduced weight gain in HFD-induced obese mice and also decreased the amount of WAT in both control diet- and HFD-fed mice without affecting food intake. Further, the SHFD group exhibited potential POMC/CART pathway dysregulation, whereas the PHFD group did not, suggesting that the appetite-suppressing pathway may not be impaired in the PHFD group. The higher fecal NEFA excretion, the lower *Cd36* mRNA expression, and lower *Apoa4* mRNA expression tendency in Korean PNO-fed mice indicated that Korean PNO may have lower intestinal lipid absorption efficiency. The lower *Lpl* mRNA expression and higher *Adrb3* mRNA expression trend may suggest that Korean PNO attenuated fat accumulation in WAT by decreasing lipid uptake by adipocytes and increasing lipolysis in adipocytes.

One of the limitations of this study was that expression of gastrointestinal hormone receptors, hypothalamic neuropeptides, and genes related to lipid metabolism were exclusively determined at the gene expression level. However, the genes we measured herein have also been assessed at the protein [29,30] and phenotype [31,32] levels in other studies.

In conclusion, the gene expression results suggest that dietary Korean PNO consumption may decrease body fat accumulation and could prevent the dysregulation of the appetite-suppressing pathway induced by HFD consumption. Nevertheless, additional phenotypic analyses are needed to confirm the overall effects of Korean PNO on whole-body lipid and energy metabolism.

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