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The Effect of Korean Pine Nut Oil on the Factors Involved in Body Fat Accumulation in Obese Mice Fed High-fat Diet

고지방 식이로 유도된 비만 마우스에서 잣기름이 체지방량 조절 관련 요인에 미치는 영향

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Abstract

The Effect of Korean Pine Nut Oil on the Factors Involved in Body Fat Accumulation in Obese Mice Fed High-fat Diet

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Korean pine nut oil (PNO) has been reported to suppress appetite by increasing satiety hormone release. However, the effects of PNO on the expression of satiety hormone receptors and neuropeptides have not been studied. Also, there is limited information on whether PNO has an influence on lipid metabolism. In this study, 5-wk-old C57BL/6 mice were fed control diets containing 10% kcal fat from PNO or soybean oil (SBO) (PC or SC) or high-fat diets containing 35% kcal fat from lard and 10% kcal fat from PNO or SBO (PHF or SHF) for 12 weeks. The mRNA expression levels of cholecystokinin related genes, ghrelin related genes, neuropeptides, and genes associated with lipid metabolism in the small intestine and white adipose tissue were quantified by real-time PCR. Overall, PNO-fed mice gained less weight ($P = 0.01$) and had less white adipose tissue ($P < 0.01$)

despite no difference in daily food intake between SBO- and PNO-fed mice. PC and PHF groups had less amount of white adipose tissue compared with SC group (30% less, P = 0.05) and SHF group (18% less, P = 0.03), respectively. Altogether, PNO-fed mice had significantly higher mRNA expression of *Growth hormone secretagogue receptor* (*Ghsr*, P = 0.03) and *Agouti-related peptide* (*Agrp, P* = 0.02), and tended to have higher mRNA expression of *Pro-opiomelanocortin* (*Pomc, P* = 0.08) and *Cocaine- and amphetamine-regulated transcript* (*Cart, P* = 0.06) in hypothalamus. PC group had higher *Ghsr* mRNA expression than SC group (1.23-fold, P = 0.02). PHF group had higher *Agrp* mRNA expression than SHF group (2.16-fold, P = 0.02). Collectively, PNO-fed mice had lower mRNA expression of jejunal *Cd36* (P = 0.03) and epididymal *Lipoprotein lipase* (*Lpl*, P = 0.02). PC group had lower *Lpl* mRNA expression than SC group (38% less, P = 0.04). Overall, PNO-fed mice tended to have lower jejunal *Apolipoprotein A-IV* mRNA expression (*Apoa4, P* = 0.07) and higher epididymal *β3-adrenergic receptor* mRNA expression (*Adrb3, P* = 0.08). Higher expression of *Ghsr* and *Agrp* mRNA in PNO-fed mice indicates that PNO-fed mice received stronger signal promoting energy consumption which might be due to less amount of white adipose tissue. The tendency of higher *Pomc* and *Cart* mRNA expression in PNO-fed mice suggests that mice in SHF group might have impaired POMC/CART pathway and failed to upregulate *Pomc* and *Cart* mRNA expression despite their higher body

weight. The lower expression of *Cd36* and *Lpl* mRNA, and the tendency of lower *Apoa4* mRNA and higher *Adrb3* mRNA expression in PNO-fed mice imply that PNO was less efficiently absorbed and stored in the body than SBO, which led to less fat accumulation in PNO-fed mice. In conclusion, PNO reduced weight gain and alleviated the possibility of POMC/CART pathway dysregulation in high-fat diet-fed mice. The lower weight gain of PNO-fed mice seemed to be due to the effect of PNO on lipid metabolism.

KEY WORDS: Korean pine nut oil, High-fat diet, POMC/CART pathway, Jejunal lipid absorption, Epididymal lipid metabolism

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Contents

List of Tables

List of Figures

List of Abbreviations

βAR, β-adrenergic receptor

ADRB3, β3-adrenergic receptor

AgRP, agouti-related peptide

ApoA-IV, apolipoprotein A-IV

CART, cocaine- and amphetamine-related transcript

CCK, cholecystokinin

CCKAR, cholecystokinin A receptor

CCKBR, cholecystokinin B receptor

CD36, cluster of differentiation 36

DGAT, diacylglycerol O-acyltransferase

FABP, fatty acid binding protein

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

GHSR, growth hormone secretagogue receptor

GLP-1, glucagon-like peptide-1

GOAT, ghrelin O-acyltransferase

LCFA, long-chain fatty acid

LPL, lipoprotein lipase

NPY, neuropeptide Y

OXM, oxyntomodulin

PGC-1α, peroxisome proliferater-activated receptor γ coactivator-1α

PLIN1, perilipin 1

PNO, Korean pine nut oil

POMC, pro-opiomelanocortin

PPAR, peroxisome proliferater-activated receptor

PYY, peptide YY

UCP, mitochondrial uncoupling protein

I. INTRODUCTION

Obesity is one of the major risk factors for chronic diseases such as hypertension, dyslipidemia, type 2 diabetes, cardiovascular disease, and some cancers (Must et al. 1999). Prevalence of obesity has increased in many countries. This has triggered global interest in finding effective, safe, and easy strategies to lose weight, and many natural substances have been studied for the possible weight loss effect which could be applied for the general population.

Pine nuts have been consumed for centuries around the world, especially in the Mediterranean and Asian regions. About 60 percent of the weight of pine nuts is composed of oils. Korean pine (*Pinus Koraiensis*) nut oil contains 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). The percentage of pinolenic acid is uniquely higher in Korean pine nut oil than other pine nut oils (Wolff et al. 2000). Pinolenic acid, which is a kind of unsaturated polymethylene-interrupted fatty acids with a *cis*-5 ethylenic bond (Lee et al. 2004), is the positional isomer of γ-linolenic acid (Matsuo et al. 1996).

Most of the studies which investigated the functional properties of Korean pine nut oil have been focused on its appetite suppressing effect. Some studies showed that Korean pine nut oil increased satiety hormone release and reduced appetite in overweight post-menopausal women

(Pasman et al. 2008) and reduced food intake in overweight women (Hughes et al. 2008). However, there is no information on whether Korean pine nut oil has an influence on expression of satiety hormone receptors and neuropeptides in hypothalamus which plays an important role in the regulation of food intake and energy expenditure (Sainsbury et al. 2002). In addition, no study showed whether appetite suppressing effect of Korean pine nut oil leads to body weight change. Moreover, the effect of Korean pine nut oil was studied in the form of supplementation not as a part of dietary consumption.

Previous studies have demonstrated that maritime pine (*Pinus pinaster*) seed oil, which contains 7% pinolenic acid (Asset et al. 1999), lowered plasma cholesterol and phospholipid in mice expressing human apolipoprotein B (Asset et al. 2001) and in apolipoprotein E-deficient mice (Asset et al. 2000). These findings showed that Korean pine nut oil can affect lipid metabolism.

Therefore, in the present study, we aimed to investigate the effects of Korean pine nut oil (PNO) enrichment in the diet on weight gain, appetite control, and lipid metabolism in C57BL/6 mice fed high-fat diets (45% kcal fat) or control diets (10% kcal fat from PNO or SBO) for 12 weeks.

II. LITERATURE REVIEW

1. Gastrointestinal regulation of energy homeostasis

The gastrointestinal organs and associated visceral organs, including the pancreas, liver, and adipose tissue, play a significant role in the regulation of energy homeostasis. The organs communicate with the hypothalamus, which regulates food intake and energy expenditure, through neural and endocrine pathways (Badman et al. 2005). Gastrointestinal hormones influence energy intake by stimulating satiety or hunger. Cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM) and peptide YY (PYY) are major gut hormones act to increase satiety and decrease food intake (Chaudhri et al. 2006). Ghrelin is the prototypical appetite-stimulating gut hormone (Briggs et al. 2010).

Cholecystokinin (CCK)

CCK is synthesized in the I-cells of duodenum and jejunum. It is also abundantly found in the central nervous system and functions as a neurotransmitter. Its secretion is stimulated by dietary fat, protein, or the products of their digestion. CCK reduces meal size and duration, but its effect is short-lived (Chaudhri et al. 2006).

Two receptors for CCK which have been identified are distributed throughout the central nervous system and gut; however, CCK A receptors

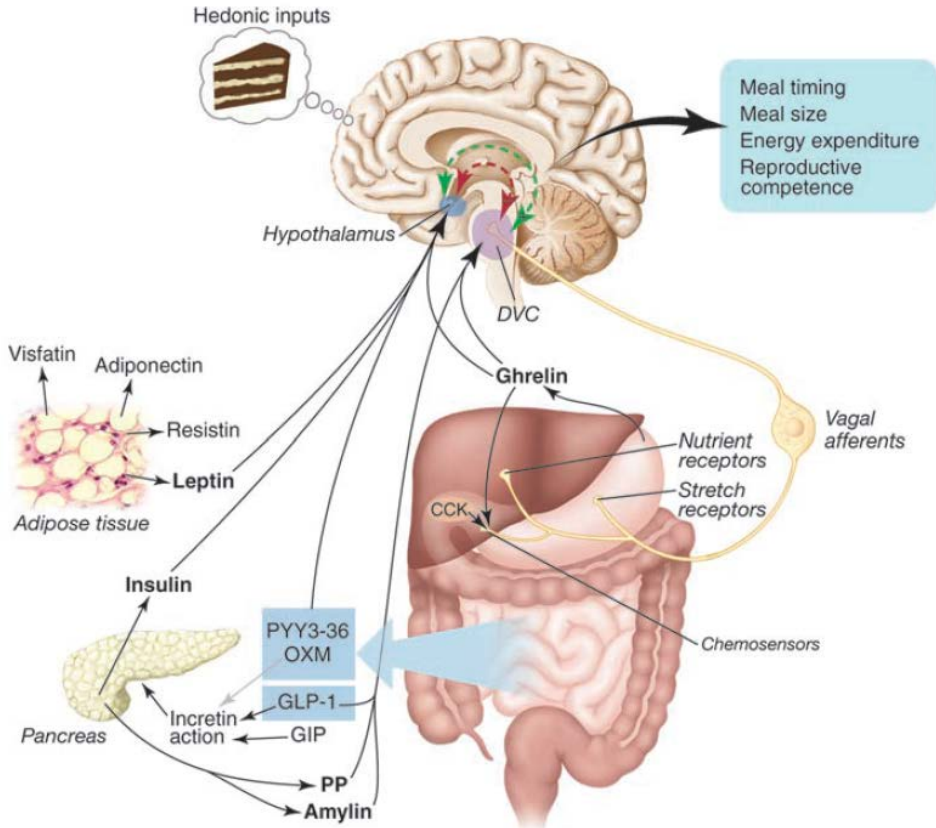


Figure 1. Peripheral signals involved in energy balance[1] (Badman et al. 2005).

[1]CCK, cholecystokinin; DVC, dorsal vagal complex; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; OXM, oxyntomodulin; PP, pancreatic polypeptide; PYY, peptide YY.

are prevalent in the alimentary tract, and CCK B receptors are prevalent in the brain. CCK promotes gallbladder contraction, increases secretion of pancreatic enzymes and bicarbonate, inhibits gastric acid secretion, and slows gastric emptying via CCK A receptors. CCK B receptors have been reported to correlate with anxiety and depression, and other central nervous system actions of CCK (Wank 1995).

The mechanism of CCK for hunger suppression is still controversial. Some studies have suggested that the inhibitory effect of CCK on gastric emptying may contribute feeding inhibition, and CCK may stimulate gastric mechanoreceptors, which induces neural feedback to the brain. However, other studies have shown that CCK reduces food intake through nongastric pathways (Moran et al. 1988).

Glucagon-like peptide-1 (GLP-1)

GLP-1 is cleaved from the transcription product of the proglucagon gene. It is mainly released from L-cells of distal small and large intestine in response to the presence of nutrients in the small intestine, in particular, carbohydrate and fat. In common with other gut peptides, GLP-1 also acts as a neurotransmitter in central nervous system, and decreases appetite and caloric intake (Gutzwiller et al. 1999).

Major actions of GLP-1 is promoting insulin secretion, suppressing glucagon release, inhibiting gastric emptying and gastric acid secretion, and

increasing pancreatic β-cell mass (Holst 2005).

The GLP-1 receptors, which are a G-protein-coupled and seven-transmembrane domain protein, are distributed in a number of areas of the brain important in appetite control, such as the hypothalamus and the brainstem (Wei et al. 1995).

There are two scenarios regarding the pathways through with GLP-1 reduces food intake. GLP-1 produced in the central nervous system may act on a circuit that mediates appetite in the brain, or GLP-1 released into the circulation after a meal may access to important regions of the hypothalamus and the brainstem and subsequently induce a feeling of satiety. These are not mutually exclusive, and an integrative model could explain the importance of the vagus nerve and the brainstem-hypothalamic connections (Chaudhri et al. 2006).

Oxyntomodulin (OXM)

In common with GLP-1, OXM is also made from preglucagon gene product and released from L-cells in response to nutrients in the form of fat or carbohydrate. It inhibits gastric motility and gastric acid secretion, and promotes insulin secretion (Schjoldager et al. 1989).

OXM potently inhibits food intake and suppresses appetite with the assistance of GLP-1 like receptors, but the affinity is lower than GLP-1. However, unlike GLP-1, which activates cells in the brainstem and other

brain regions, the stimulation of OXM is limited to the hypothalamus (Dakin et al. 2004). The mechanism by which OXM controls appetite may involve suppression of appetite-stimulating hormone ghrelin (Cohen et al. 2003).

Peptide YY (PYY)

PYY is mainly expressed in the L-cells of ileum and large intestine, and also identified in the central nervous system, including hypothalamus, medulla, and pons. It is released in response to a meal in relation to the meal composition; its release is highly promoted by isocaloric meals of fat compared with meals consisting of protein or carbohydrate. Intraluminal bile acids, gastic acid, and CCK stimulate PYY release, as well (Chaudhri et al. 2006).

PYY contributes to the ‘ileal brake’ effect, which inhibits further food intake when nutrients have reached the distal small intestine. PYY delays gastic emptying and gallbladder emptying, mediated by the vagus nerve. It decreases the expression of orexigenic neuropeptides and increases that of anorexigenic neuropeptides in the hypothalamus (Batterham et al. 2002).

Ghrelin

Ghrelin is a peripherally active appetite-stimulating gut hormone, and its mRNA is expressed throughout the gastrointestinal tract; however, the major

source of circulating ghrelin is the A-cells of gastric fundus. It is suggested that ghrelin may be involved in meal initiation since its level rise during fasting and fall on eating (Chaudhri et al. 2006).

Ghrelin, which is the ligand for growth hormone secretagogue receptor, induces growth hormone and other pituitary hormone secretion from the anterior pituitary. It also increases gastric motility and promotes pancreatic polypeptide release. Its levels are inversely correlated with body weight, which explains why diet-induced weight loss is difficult to be maintained. The lower ghrelin level in obesity is thought to be a feedback mechanism to reduce appetite (Cummings et al. 2002).

2. Hypothalamic regulation of energy homeostasis

The integrated regulation of food intake and energy expenditure occurs in the hypothalamic regions of the brain. In the hypothalamus, two major groups of neuropeptides involved in orexigenic and anorexigenic processes exist. Neuropeptide Y (NPY) and agouti-related peptide (AgRP) are the appetite-stimulating neuropeptides, and pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) are the appetite-suppressing neuropeptides. To regulate food intake and energy expenditure, the neurons expressing these neuropeptides interact with signals from the periphery and with each other. When these systems are dysregulated, obesity often develops (Sainsbury et al. 2002).

Neuropeptide Y (NPY) & Agouti-related peptide (AgRP)

NPY and AgRP, which increase food intake and body energy stores, are expressed in the arcuate nucleus in the hypothalamus. Their expression is stimulated by food deprivation or on low-energy diets and is affected by changes in various hormones (Woods et al. 1998). The levels of these peptides rise in response to elevated levels of glucocorticoids and ghrelin, and reduced levels of leptin and insulin (Leibowitz et al. 2004).

In negative energy balance, NPY and AgRP stimulate food intake, reduce glucose and lipid utilization, and promote de novo lipogenesis. The anabolic effects of NPY are facilitated by local γ-amino butyric acid (GABA)

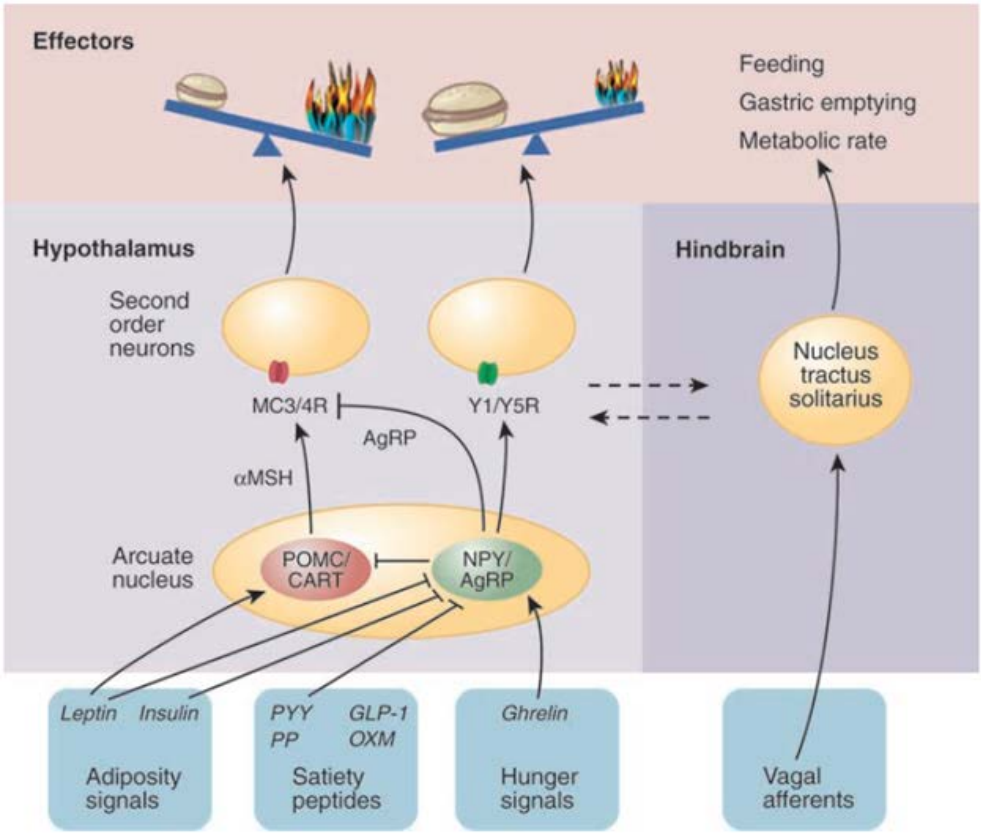


Figure 2. Potential action of peripheral signals on the hypothalamus[1] (Badman et al. 2005).

[1]αMSH, α melanocyte-stimulating hormone; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; MC3/4R, melanocortin 3 and melanocortin 4 receptors; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; Y1/Y5R, Y1 and Y5 receptors.

neurons, which suppress the melanocortin neurons and their catabolic actions. These two peptides, NPY and AgRP, are evolved to guarantee the hunger signals during energy deficiency and enable the body to endure long periods of negative energy balance (Leibowitz et al. 2004).

Pro-opiomelanocortin (POMC) & Cocaine- and amphetamine-related transcript (CART)

POMC and CART are synthesized in the ARC, and POMC/CART neurons show the same distribution pattern as NPY/AgRP neurons. Their expression is stimulated under conditions of positive energy balance, in association with excess calorie intake, elevated leptin and insulin levels, and reduced levels of ghrelin (Leibowitz et al. 2004). However, there is a big difference between two peptides, which is POMC is not stimulated by high-fat diet consumption whereas CART is stimulated by signals related to dietary fat and increased circulating lipids (Wortley et al. 2004).

POMC and CART attenuate food intake and stimulate sympathetic nervous system to promote metabolic effects, including thermogenesis. Through this mechanism, they prevent excess body fat accumulation during high caloric intake (Leibowitz et al. 2004).

3. Lipid absorption in the small intestine

Dietary fats, which provide essential fatty acids and fat-soluble vitamins, are important for health. However, increased fat consumption and changed dietary fat composition have significantly contributed to the increase in obesity, which once more provoked a big interest in the role of the small intestine in regulating lipid homeostasis (Abumrad et al. 2012).

Cellular long-chain fatty acid uptake

Long-chain fatty acids (LCFA) are the major components of the dietary lipids. LCFA uptake by cells takes place by both spontaneous and facilitated transfer. Passive diffusion occurs as three successive steps. LCFA is adsorbed on the membrane surface, moved from external hemi-leaflet of bilayer to internal hemi-leaflet, and desorbed from internal bilayer into the inner of vesicle. Facilitated diffusion occurs by the brush border membrane lipid-binding proteins: the fatty acid transporter (CD36), the plasma membrane-associated fatty acid-binding protein, and the fatty acid transport protein 4 (Niot et al. 2009). CD36 is a multiligand transmembrane protein, expressed abundantly in the duodeno-jejunum. Intestinal CD36 gene expression has been shown to parallel dietary lipids contents. It also plays a key role in incorporation of LCFA into TG for chylomicron production (Drover et al. 2005).

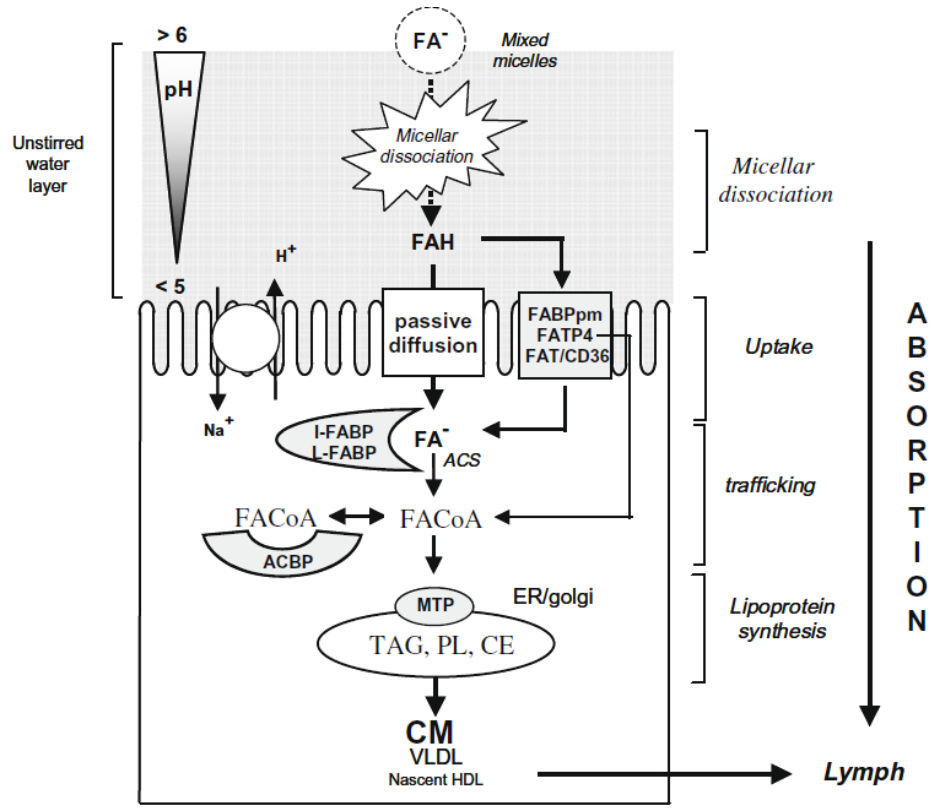


Figure 3. Intestinal lipid absorption mechanism[1] (Niot et al. 2009).

[1]ACBP, acyl-CoA-binding protein; ACS, acyl-CoA synthetases; CE, cholesterol esters; CM, chylomicrons; ER, endoplasmic reticulum; FA^-, ionized long-chain fatty acids; FABPpm, plasma membrane fatty acidbinding protein; FAH, protonated long-chain fatty acids; FATP4, fatty acid transport protein 4; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; MTP, microsomal triacylglycerol transfer protein; PL, phospholipids; TAG, triacylglycerol (triglycerides); VLDL, very low density lipoproteins.

Intracellular trafficking of long-chain fatty acid uptake

Once entering the small intestinal enterocyte, LCFA and fatty acid acyl-CoA are bound to fatty acid-binding proteins (FABP) and acyl-CoA binding protein, respectively. In the intestine, two types of the FABP, intestinal-type FABP and liver- type FABP, are expressed. However, their properties and distributions in the intestinal tract are not identical. They deliver fatty acids from phospholipid membranes by different mechanisms; intestinal-type FABP uses collision whereas liver-type FABP uses diffusion. Also, intestinal-type FABP binds one fatty acid molecule while liver-type FABP binds two fatty acid molecules as well as other lipids, including cholesterol, acyl-CoA, and monoacylglycerol. Moreover, intestinal-type FABP targets fatty acids toward TG synthesis; however, LFABP directs fatty acids toward oxidation (Lagakos et al. 2011).

Chylomicron assembly and trafficking

Fatty acid acyl-CoA is re-esterified in TG in the endoplasmic reticulum membrane. The terminal step in TG synthesis is catalyzed by diacylglycerol acyltransferase (DGAT) which transfers fatty acid acyl-CoA to DG. Two types of DGAT are expressed in the intestine: DGAT1 and DGAT2. DGAT1 null mice have reduced chylomicron secretion and are resistant to high-fat induced obesity. Otherwise, DGAT2 knockout mice are reported to die shortly after birth due to undernutrition and loss of skin barrier function.

These reports imply that DGAT2 is more essential for TG synthesis than DGAT1 in most tissues other than intestine (Abumrad et al. 2012).

TG molecules are delivered into endoplasmic reticulum cisternae by the carnitine acyl-transferase-like system and the microsomal triacylglycerol transfer protein which plays a significant role in lipoprotein synthesis. TG molecules are used to form pre-chylomicrons. Apolipoprotein A-IV (Apo A-IV), synthesized by enterocytes during lipid ingestion, incorportates to the nascent chylomicron and stabilizes the particle. Pre-chylomicrons are transferred from endoplasmic reticulum to the Golgi apparatus in which the final maturation of chylomicron takes place. Chylomicrons move from the intestinal mucosa into the lymphatic system, and then enter the blood (Niot et al. 2009).

4. Lipid metabolism in the white adipose tissue

The white adipose tissue (WAT) is the predominant type of adipose tissue. It stores excess dietary calories as TG within lipid droplets, and secretes adipokines, such as leptin and adiponectin, to regulate energy balance. Accordingly, understanding lipid metabolism in the WAT is important to ameliorate or prevent obesity (Marcelin et al. 2010).

Storage and mobilization of fats

The blood carries chylomicrons to white adipose tissue. In the capillaries of white adipose tissue, the extracellular enzyme lipoprotein lipase (LPL) hydrolyzes TG to fatty acids and glycerol, which are taken up by adipocytes. They are re-esterified for storage as TG in lipid droplets within adipocytes (Davies et al. 2012).

The surface of lipid droplets in adipocytes is coated with perilipins, a family of phosphoproteins that restrict access to lipid droplets, preventing unexpected lipid mobilization. Epinephrine and glucagon, released in response to low blood glucose levels, activate adenylyl cyclase which produces cyclic AMP. Cyclic AMP-dependent protein kinase phosphorylates perilipin, and phosphorylated perilipin causes hormone-sensitive lipase in the cytosol to move to the lipid droplet surface, where it can hydrolyze TG to fatty acids and glycerol (Large et al. 2004).

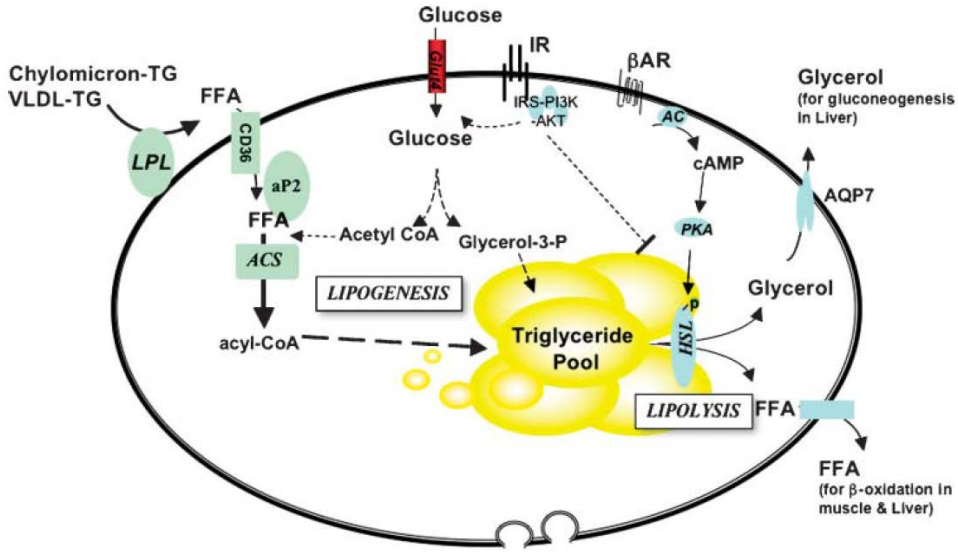


Figure 4. Lipid metabolism in adipocytes[1] (Sethi et al. 2007)

[1]AC, adenylate cyclase; ACS, acyl-CoA synthase; AKT, AKR mouse thymoma viral proto-oncogene; AR, adrenergic receptor; HSL, hormone sensitive lipase; IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A.

Regulation of fat mass

Adipokines produce changes in fuel metabolism and feeding behavior that hold the amount of adipose tissue at a suitable level (Marcelin et al. 2010). Leptin, the primary adipokine, reduces energy intake and increases energy expenditure. It stimulates sympathetic nervous system which controls hydrolysis of stored energy and adaptive thermogenesis. Fuel utilization is regulated by β-adrenergic receptors (βARs) which are a class of G protein-coupled receptors. The binding of βARs to the G-proteins elevates intracellular cAMP level and activates PKA, which is responsible for activating lipolytic enzymes (Collins et al. 2010). Among the three subtypes of βARs, β3-ARs are reported to have a significant effect on insulin secretion, food intake, and energy expenditure (Grujic et al. 1997).

Adaptive thermogenesis is involved in uncoupling the mitochondria. Mitochondrial uncoupling protein (UCP) allows continual oxidation of fatty acids without ATP synthesis. Whereas UCP1 is highly expressed in the brown adipose tissue, UCP2 is widely expressed in both central and peripheral tissues involved in glucose and lipid metabolism. UCP2 participates in the regulation of food intake, insulin secretion, and immune responses (Diano et al. 2011).

Dietary fatty acids and their metabolic derivatives regulate the expression of genes central to regulate lipid homeostasis, including peroxisome proliferater-activated receptors (PPARs) which are a family of

ligand-activated transcription factors. The three PPARs, PPARα, PPARγ, and PPARδ, have different distribution patterns and functions (Evans et al. 2004).

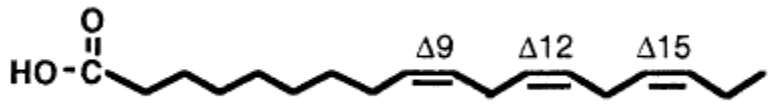
PPARα is primarily expressed in liver and promotes fatty acid oxidation during fasting in order to generate ketone bodies which is an energy source for peripheral tissues. It also acts to lower plasma triglycerides, reduce adiposity, and improve hepatic and muscle steatosis. PPARγ is mainly expressed in liver and adipose tissue. It regulates the formation of fat cells and their ability to store lipids, and its expression is promoted during adipocyte differentiation. PPARδ is expressed abundantly throughout the body but not in liver. It stimulates fat-buring by inducing the transcription of genes involved in fatty acid catabolism and thermogenesis. Many effects of PPARγ coactivator-1α (PGC-1α) is mediated through PPARδ. PGC-1α induces genes required for mitochondrial biogenesis and uncoupling (Evans et al. 2004).

5. Characteristics of pine nut oil

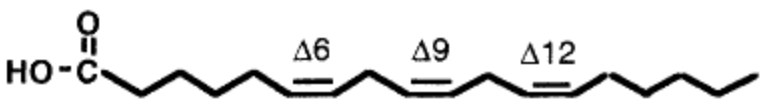
Pine nuts, the edible seeds of pines, have been eaten in Europe and Asia. They are produced by about 20 species of pine, including Korean pine (*Pinus koraiensis*), maritime pine (*Pinus pinaster*), and stone pine (*Pinus pinea*). Korean pine is the most important species in international trade and widely harvested in northeast Asia. Maritime pine is a pine native to the western and southwestern Mediterranean region. Stone pine is produced in Europe (Wolff et al. 1995).

According to the Korean food composition table (2011), pine nuts contain 17.6% of carbohydrate, 15.4% of proteins, and 61.5% of fats. Pine nuts can be pressed to extract pine nut oil. Pine nut oil has been used for culinary purposes in European and American regions and for medicinal uses in Asian regions.

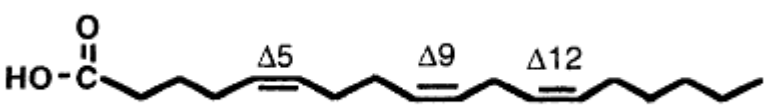
Korean pine nut oil has been reported to contain 4% palmitic acid (16:0), 28% oleic acid (18:1, Δ9), 47% linoleic acid (18:2, Δ9,12), and 14% pinolenic acid (18:3, Δ5,9,12). Korean pine nut oil uniquely has much higher percentage of pinolenic acid than other pine nut oils (Wolff et al. 2000). Maritime pine nut oil contains 7% pinolenic acid (Asset et al. 1999), and stone pine nut oil contains 1% pinolenic acid (Pasman et al. 2008). Pinolenic acid, which is a kind of unsaturated polymethylene-inturrupted fatty acids with a cis-5 ethylenic bond (Lee et al. 2004), is the positional isomer of γ-linolenic acid (Matsuo et al. 1996).



α-Linolenic acid (*cis*-9,*cis*-12,*cis*-15-Octadecatrienoic acid)



γ-Linolenic acid (*cis*-6,*cis*-9,*cis*-12-Octadecatrienoic acid)



Pinolenic acid (*cis*-5,*cis*-9,*cis*-12-Octadecatrienoic acid)

Figure 5. Structures of α- and γ-Linolenic acids, and pinolenic acid (Ogawa et al. 2005).

Korean pine nut oil has been reported to reduce appetite and food intake, but the results have been inconsistent. In a study of Pasman et al (2008), Korean pine nut oil stimulated CCK and GLP-1 secretion and decreased appetite in overweight women. Also, Korean pine nut fatty acids produced CCK release in STC-1 cell which is a murine neuroendocrine tumor cell line. In particular, Korean pine nut fatty acids were 8-fold more potent in releasing CCK than stone pine nut fatty acids. The authors explained that the difference in pinolenic acid concentration may be the reason for the discrepancy in CCK release. Hughes et al (2008) also showed that Korean pine nut fatty acids reduced food intake in overweight women. In detail, 2g of Korean pine nut fatty acids provided 30 minutes before ad-libitum lunch reduced grams of food intake by 9%, corresponded to 50 kcal reduction in energy intake compared to olive oil.

However, Verhoef et al (2011) recently reported that Korean pine nut TG has no effects on satiety and energy intake. In the study, 130g of yogurt with either Korean pine nut oil or milk fat were given as a breakfast to healthy women. After 4 hours, appetite profile ratings and energy intake were determined; however, there were no significant difference of appetite profile ratings and energy intake between Korean pine nut oil group and milk fat group. Accordingly, to confirm the efficacy of Korean pine nut oil as an appetite suppressant, further studies with various designs need to be researched.

While Korean pine nut oil has been focused on its appetite suppressing properties, maritime pine nut oil has been focused on its lipid lowering properties. Korean pine nut oil and maritime pine nut oil have similar fatty acid profiles; however, they have different concentration of pinolenic acid. Whereas Korean pine nut oil contains 14% pinolenic acid, maritime pine nut oil contains 7% pinolenic acid and 7% sciadonic acid (Asset et al. 1999).

Compared to lard, maritime pine nut oil decreased plasma cholesterol and phospholipid levels and increased plasma TG level in apolipoprotein E-deficient mice (Asset et al. 2000). In mice expressing human apolipoprotein B, maritime pine nut oil lowered plasma cholesterol, TG, and phospholipid levels relative to coconut oil (Asset et al. 2001). In apolipoprotein E-deficient mice, plasma total cholesterol level was lower and plasma TG was higher in mice fed maritime pine nut oil than in those fed lard (Asset et al. 1999).

III. MATERIALS AND METHODS

1. Animals and diets

Male C57BL/6N mice, aged 5 weeks, were purchased from Central laboratory animal Inc. (Seoul, Korea) and maintained on a chow diet for 3 days before divided into 4 dietary groups; control diets containing 10% kcal fat from PNO or SBO (PC or SC) or high-fat diets containing 35% kcal fat from lard and 10% kcal fat from PNO or SBO (PHF or SHF). Animals were fed experimental diets *ad libitum* for 12 weeks. **Table 1** shows the composition of the experimental diets. The fatty acid composition of the experimental diets is shown in **Table 2**. PNO used in the experiment was a gift from Dubio Co., Ltd. (GyeongGi-do, Korea). All mice were housed individually with controlled temperature (23 ± 3 °C), humidity (55 ± 10 %), and a 12 hour-dark/light cycle. Body weight and food intake of the mice were measured once and 4 times a week, respectively. At the end of the experimental period, mice were euthanized with CO_2 asphyxiation after 12 hour fasting. Brain, stomach, small intestine, and white adipose tissues (epididymal, subcutaneous, and perirenal-retroperitoneal fat depots) were removed, and white adipose tissues were weighed. Hypothalamus was dissected from the brain using surgical blades. Stomach was opened along the greater curvature and washed in phosphate-buffered saline. After contents were removed, small intestine was divided into 3 parts: duodenum

(the first 2 cm of the small intestine), ileum (the last 2 cm of the small intestine), and jejunum (rest of the small intestine). All tissues were snap-frozen in liquid nitrogen, and stored at -80°C until subsequent analysis. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee of Seoul National University (approval no. SNU-101029-1).

Table 1. Composition of the experimental diets[1]

	Control (10% Kcal Fat)	High-fat (45% Kcal Fat)
Casein (g)	200	200
L-cystine (g)	3	3
Sucrose (g)	350	172.8
Cornstarch (g)	315	72.8
Dyetrose (g)	35	100
Pine nut oil[2] or Soybean oil (g)	45	45
Lard (g)	0	157.5
t-Butylhydroquinone (g)	0.009	0.009
Cellulose (g)	50	50
Mineral Mix[3] (g)	35	35
Vitamin Mix[4] (g)	10	10
Choline Bitartrate (g)	2	2
Total (g)	1045.0	848.1
Kcal/ g diet	3.69	4.64

[1]Resource : Dyets, Inc., Bethlehem, PA, USA

[2]Pine nut oil was a gift from Dubio, Co., Ltd. (GyeongGi-do, Korea)

[3]Thirty five grams of mineral mix (Dyets, #210099) provides 1.0g sodium, 1.6g chloride, 0.5g magnesium, 0.33g sulfur, 59mg manganese, 45mg iron, 29mg zinc, 6mg copper, 2mg chromium, 1.6mg molybdenum, 0.16mg selenium, 0.9mg fluoride, 0.2mg iodine and 3.99g sucrose.

[4]Ten grams of vitamin mix (Dyets, #300050) provides 4000IU vitamin A, 1000IU vitamin D_3, 50IU vitamin E, 30mg niacin, 16mg pantothenic acid, 7mg vitamin B_6, 6mg vitamin B_1, 6mg vitamin B_2, 2mg folic acid, 0.5mg menadione, 0.2mg biotin, 10ug vitamin B_{12} and 9.78 sucrose.

Table 2. Fatty acid composition of the experimental diets[1] (% of fat)

	Control		High-fat	
	SC	PC	SHF	PHF
Myristic acid (C14:0)	.	.	0.9	0.9
Palmitic acid (C16:0)	11.9	7.0	18.9	17.8
Stearic acid (C18:0)	4.8	3.6	11.1	10.7
Total SFA	16.7	10.6	30.9	29.4
Palmitoleic acid (C16:1 Δ9)	.	.	1.4	1.4
Oleic acid (C18:1 Δ9)	21.1	27.4	34.7	36.0
Total MUFA	21.1	27.4	36.1	37.4
Linoleic acid (C18:2 Δ 9,12)	54.9	47.2	30.3	28.6
α-linolenic acid (C18:3 Δ9,12,15)	7.4	0.8	2.8	1.3
Pinolenic acid (C18:3 Δ 5,9,12)	.	14.0	.	3.3
Total PUFA	62.3	62.0	33.1	33.2

[1]Total lipids were extracted from the experimental diet using a Folch extraction protocol (Folch et al. 1957). Extracted lipids were saponified with sodium hydroxide in methanol and methylated in the presence of boron trifluoride in methanol at 100°C. Fatty acid methyl esters were extracted with hexane, and 1 μL aliquots of the extracts were injected in to a GC Agilant 7890A (Agilent, CA, USA) equipped with a flame ionization detector in the split mode (1:10). Helium was used as a carrier gas at a flow rate of 1.5 mL/min. DB-carbowax (0.32mm × 25m, 0.2μm, Agilent) was used as a capillary column. The oven temperature was increased from 50°C to 220°C at a rate of 15°C/min and held at maxium temperature for 20min.

2. Determination of serum leptin concentration

Blood was collected via heart puncture and serum was separated by centrifugation at 1,000 rpm for 20 min at 4°C after 2 hour clotting. Serum leptin concentration was measured by Quantikine® ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instruction. In detail, 50 μL of 20-fold diluted serum or standard and 50 μL of assay diluent were added to each well of 96-well plates coated with a polyclonal antibody specific for mouse leptin. After incubated for 2 hours at room temperature, each well was washed for 5 times with 400 μL of wash buffer. Subsequently, 100 μL of a polyclonal antibody against mouse leptin conjugated to horseradish peroxidase (HRP) was added and incubated for 2 hours at room temperature. After 5 times of washes, 100 μL of substrate solution was added and incubated for 30 minutes at room temperature with protection from light. Lastly, 100 μL of diluted hydrochloric acid solution was added to stop the reaction. Within 30 minutes, the optical density of each well was determined using a microplate reader (Spectramax 190, Molecular Devices, CA, USA) set to 450nm and 570nm. The leptin concentration was calculated by subtracting the readings at 570nm from those at 450nm.

3. Determination of serum triglyceride and cholesterol concentrations

Serum TG concentration was determined using commercial kit (Asan Pharmaceutical, Korea) based on enzymatic assay. The enzyme mixture in the solution hydrolyzes TG to glycerol and fatty acids, phosphorylates glycerol into glycerophosphoric acid, and oxidizes glycerophosphoric acid. Oxidation of glycerophosphoric acid creates hydrogen peroxide which produces quinoid dyes by reacting with 4-aminoantipyrine, N-ethyl-N-sulfopropyl-m-toluidine, and peroxidase. Serum TG content was calculated based on the absorbance of quinoid dyes. In this study, 2 μL of serum or standard (300 mg/dL of glycerol) and 300 μL of the enzyme solution were added to each well of 96-well plates and incubated for 10 minutes at 37°C. The absorbance was measured using a microplate reader (Spectramax 190, Molecular Devices, CA, USA) set to 550nm.

Serum cholesterol concentration was determined using commercial kit (Asan Pharmaceutical, Korea) based on enzymatic assay. The enzyme mixture in the solution hydrolyzes esterified cholesterol to free cholesterol and fatty acids, and oxidizes free cholesterol into Δ^4-cholestenone and hydrogen peroxide. The hydrogen peroxide creates quinone dyes by reacting with 4-aminoantipyrine, phenol, and peroxidase. Serum cholesterol content was calculated based on the absorbance of quinone dyes. In this study, 2 μL

of serum or standard (300 mg/dL of esterified cholesterol) and 300 μL of enzyme solution were added to each well of 96-well plates and incubated for 5 minutes at 37°C. The absorbance was measured using an identical microplate reader used in quantification of serum TG level set to 500nm.

4. RNA extraction and cDNA synthesis

Trizol reagent (Invitrogen, CA, USA) was used to extract total RNA from hypothalamus, rest of the brain, stomach, jejunum, and epididymal fat tissue according to the manufacturer's instructions. Hundred mg of epididymal fat or 50 mg of other tissues were homogenized in 1 mL of Trizol reagent using a power homogenizer (IKA T10 Basic Ultra-turrax, IKA, Germany), and the homogenized samples were incubated for 5 minutes at room temperature. After 0.2 mL of chloroform was added, the homogenized samples were shaken vigorously by vortexing for 15 seconds, incubated for 3 minutes at room temperature, and centrifuged at 12,000 × g for 15 minutes at 4°C. The colorless aqueous phase was transferred to the fresh tubes and mixed with 0.5 mL of isopropyl alcohol to precipitate the RNA. After incubated for 10 minutes at room temperature, the samples were centrifuged at 12,000 × g for 10 minutes at 4°C. Supernatant was removed, and 1 mL of 75% ethanol was added to wash the RNA pellet. After mixed by vortexing, the sample was centrifuged at 7,500 × g for 2 minutes at 4°C. Subsequently, supernatant was discarded, and the RNA pellet was dried. The RNA pellet was redissolved in 20 μL of diethylpyrocarbonate (DEPC)-treated water. The concentrations of the RNA solutions were quantified using a spectrophotometer (DU530, Beckman, CA, USA), and the qualities of the RNA samples are checked by agarose gel electrophoresis using Gel-Doc XR system (Bio-Rad, CA, USA).

The cDNA was synthesized from 2 μg of total RNA with PrimeScript II 1st strand cDNA synthesis kit (Takara, Japan) using Thermal Cycler 2720 (Applied Biosystems, CA, USA). The condition for reverse transcription was 42°C for 50 minutes, 95°C for 5 minutes, and 4°C for 30 minutes.

5. Quantification of the gene expression

To identify the effects of PNO on appetite control pathway, the mRNA expression levels of cholecystokinin related genes (jejunal *Cck*, cerebral *Cckar* and *Cckbr*), ghrelin related genes (gastric *Ghrelin* and *Goat*, and hypothalamic *Ghsr*), and neuropeptides (hypothalamic *Npy*, *Agrp*, *Pomc*, and *Cart*) were quantified. To determine the influence of PNO on lipid metabolism, the mRNA expression levels of genes associated with lipid metabolism in small intestine (jejunal *Cd36*, *Ifabp*, *Dgat2*, and *ApoA4*) and those in white adipose tissue (epididymal *Lpl*, *Plin1*, *Ucp2*, *Adrb3*, *Pparg*, *Ppargc1a*, and *Ppard*) were quantified by real-time PCR with a SYBR Premix Ex Taq (Takara, Japan) and StepOne Real-time PCR System (Applied Biosystems, CA, USA).

All reactions were performed in total of 20 μL reaction volume containing 1 μL of 2 ng/μL reverse-transcribed cDNA, 10 μL of SYBR Premix Ex Taq, 0.4 μL of 10uM forward primer, 0.4 μL of 10uM reverse primer, 0.4 μL of ROX reference dye, and 7.8 μL of autoclaved distilled water. Condition for the PCR reactions were 95°C for 30 seconds to initiation, 95°C for 5 seconds and 60°C for 30 seconds up to 40 cycles. After the PCR reactions, melting curve analyses were carried out at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds in order to assess the dissociation-characteristics of double-stranded DNA during heating. The threshold cycle (Ct) values, the number of PCR cycles at which the

fluorescence signal during the reactions reaches a fixed threshold, were analyzed using StepOne^{TM} Software version 2.1 (Applied Biosystems, CA, USA). To normalize the results, ΔCt was calculated by subtracting the Ct value of house-keeping gene *Gapdh* from that of interest gene; and ΔΔCt was determined by subtracting the ΔCt value of the control group from that of experimental groups. The relative expression of genes was calculated from $2^{-\Delta\Delta Ct}$. Specific primer sequences used in this study are shown in **Table 3** and **Table 4**.

Table 3. The primer sequences used for quantification of expression of genes involved in appetite control[1]

Gene	Forward primer	Reverse primer
Cck	TCC AGC AGG TCC GCA AA	CCA GGC TCT GCA GGT TCT TAA
Cckar	ATA AAA GTT GGA GTA TTG TGT GAG CTT C	TTA AGT GTT TTC AAC ACT AAT TTT GCA
Cckbr	TGG GAC CTA ACC CTA CTC CGG TGA T	CAA ATG AGA GGG TGT ACT CAG GA
Ghrelin	TCC AAG AAG CCA CCA GCT AA	AAC ATC GAA GGG AGC ATT GA
Goat	ATT TGT GAA GGG AAG GTG GAG	CAG GAG AGC AGG GAA AAA GAG
Ghsr	ACC GTG ATG GTA TGG GTG TCG	CAC AGT GAG GCA GAA GAC CG
Npy	TCC GCT CTG CGA CAC TAC AT	TGC TTT CCT TCA TTA AGA GGT CTG
Agrp	AGC TTT GGC GGA GGT GCT	GCC ACG CGC AGA ACG A
Pomc	TGA ACA TCT TTG TCC CCA GAG A	TGC AGA GGC AAA CAA GAT TGG
Cart	GCC AAG GCG GCA ACT C	TCT TGC AAC GCT TCG ATC TG
Gapdh	GGA GAA ACC TGC CAA GTA	AAG AGT GGG AGT TGC TGT TG

[1]*Cck*, Cholecystokinin; *Cckar*, Cholecystokinin A receptor; *Ccrbr*, Cholecystokinin B receptor; *Goat*, Ghrelin O-acyltransferase; *Ghsr*, Growth hormone secretagogue receptor; *Npy*, neuropeptide Y; *Agrp*, Agouti-related peptide; *Pomc*, Pro-opiomelanocortin; *Cart*, Cocaine- and amphetamine-regulated transcript; *Gapdh*, Glyceraldehyde 3-phosphate dehydrogenase.

Table 4. The primer sequences used for quantification of expression of genes involved in lipid metabolism[1]

Gene	Forward primer	Reverse primer
Cd36	CCA AGC TAT TGC GAC ATG ATT	TCT CAA TGT CCG AGA CTT TTC A
Ifabp	AGA GGA AGC TTG GAG CTC ATG ACA	TCG CTT GGC CTC AAC TCC TTC ATA
Dgat2	TGG GTC CAG AAG AAG TTC CAG AAG TA	ACC TCA GTC TCT GGA AGG CCA AAT
Apoa4	TTC CTG AAG GCT GCG GTG CTG	CTG CTG AGT GAC ATC CGT CTT CTG
Lpl	TTA TCC CAA TGG AGG CAC TTT C	CAC GTC TCC GAG TCC TCT CTC T
Plin1	CAT CTC TAC CCG CCT TCG AA	TGC TTG CAA TGG GCA CAC TG
Ucp2	CAG GTC ACT GTG CCC TTA CCA	CAC TAC GTT CCA GGA TCC CAA
Adrb3	ACC AAC GTG TTC GTG ACT	ACA GCT AGG TAG CGG TCC
Pparg	CAG CAG GTT GTC TTG GAT GTC	AGC CCT TTG GTG ACT TTA TGG
Ppargc1a	CCG TAA ATC TGC GGG ATG ATG	CAG TTT CGT TCG ACC TGC GTA A
Ppard	AGC CAT ATT CCC AGG CTG TCT C	CCT AGG CAG CAC AAG GGT CAT
Gapdh	GGA GAA ACC TGC CAA GTA	AAG AGT GGG AGT TGC TGT TG

[1] *Cd36*, Cluster of differentiation 36; *Ifabp*, Intestinal fatty acid binding protein; *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 proliferater-activated receptor gamma; *Ppargc1a*, PPAR gamma coactivator 1 alpha; *Ppard*, Peroxisome proliferator-activated receptor delta; *Gapdh*, Glyceraldehyde 3-phosphate dehydrogenase.

6. Statistical analysis

SPSS software version 19.0 (SPSS Inc., Chicago, IL) was used for statistical analyses. Significant differences were analyzed by Two-way ANOVA for the overall effects of fat amount, oil type, and the interaction between the two followed by Fisher's LSD multiple comparison test for individual group comparisons. Differences were considered statistically significant at $P < 0.05$.

IV. RESULTS

1. Body weight changes, food intake, food efficiency, white adipose tissue weight, and serum leptin concentration

Overall, high-fat diet-fed mice had significantly higher weight gain ($P < 0.01$), white adipose tissue amount ($P < 0.01$), and serum leptin concentration ($P < 0.01$) than control diet-fed mice. Altogether, PNO-fed mice had lower weight gain ($P = 0.01$), amount of white adipose tissue ($P < 0.01$), and serum leptin concentration ($P = 0.02$) than SBO-fed mice. There were no significant difference in daily food intake and daily energy intake between SBO- and PNO-fed mice; therefore, food efficiency was lower ($P = 0.01$) in PNO-fed mice (**Table 5**).

In particular, PHF-fed mice had significantly lower body weight (10% less, $P = 0.02$), less weight gain after 12 weeks of feeding (18% less, $P = 0.02$), and lower food efficiency (17% less, $P = 0.01$) than SHF-fed mice. PC and PHF groups had significantly less amount of white adipose tissue compared with SC (30% less, $P = 0.05$) and SHF groups (18% less, $P = 0.03$), respectively. A significantly positive correlation was observed between the amount of white adipose tissue and weight gain ($r = 0.92$, $P < 0.01$). Mice in PHF group had significantly lower serum leptin level than those of SHF group (33% less, $P = 0.03$).

Table 5. Body weight, weight gain, food intake, food efficiency, white adipose tissue weight, and serum leptin concentration of the mice fed control or high-fat diets[1]

	Control		High-fat		Fat amount (*P*-value)	Oil Type (*P*-value)	Inter-action (*P*-value)
	SC	PC	SHF	PHF			
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)	32.76 ± 0.96^{ab}	30.51 ± 0.64^{a}	38.49 ± 1.45^{c}	34.58 ± 1.42^{b}	< 0.01	0.01	0.49
Body weight gain (g)	15.48 ± 0.84^{ab}	13.76 ± 0.59^{a}	21.48 ± 1.42^{c}	17.53 ± 1.31^{b}	< 0.01	0.01	0.32
Daily food intake (g)	3.20 ± 0.06^{b}	3.20 ± 0.31^{b}	2.82 ± 0.05^{a}	2.76 ± 0.04^{a}	< 0.01	0.54	0.48
Daily energy intake (kcal)	11.80 ± 0.21^{a}	11.82 ± 0.11^{a}	13.11 ± 0.22^{b}	12.82 ± 0.20^{b}	< 0.01	0.50	0.43
Food efficiency (mg/kcal)[2]	15.56 ± 0.68^{a}	13.83 ± 0.52^{a}	19.43 ± 1.11^{b}	16.18 ± 1.03^{a}	< 0.01	< 0.01	0.40
White adipose tissue (g)	3.10 ± 0.22^{b}	2.18 ± 0.18^{a}	5.34 ± 0.38^{c}	4.38 ± 0.39^{d}	< 0.01	< 0.01	0.95
Serum leptin (μg/L)	19.89 ± 2.82^{ab}	12.61 ± 1.96^{a}	43.26 ± 6.82^{c}	29.19 ± 4.52^{b}	< 0.01	0.02	0.46

Data are presented as means ± SEM, n = 10-11 for each group.

[1]Two-way ANOVA was used to determine the significant effect of fat amount and oil type. Different superscripts indicate significant differences at $P < 0.05$ by Fisher's LSD multiple comparison test.

[2]Food efficiency (mg/kcal) = Weight gain (mg) / Total food intake (kcal)

SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard

2. Serum triglyceride and cholesterol concentrations

Serum triglyceride concentration was not affected by fat amount and oil type, while serum cholesterol tended to be lower in PNO-fed mice (P = 0.09). Interaction between fat amount and oil type was not significant (**Table 6**).

Table 6. Serum triglyceride and cholesterol concentrations[1]

	Control		High-fat		Fat amount (*P*-value)	Oil type (*P*-value)	Inter-action (*P*-value)
	SC	PC	SHF	PHF			
Serum triglyceride (mg/dL)	115.96 ± 8.41	133.48 ± 10.39	165.10 ± 34.43	122.20 ± 15.31	0.36	0.54	0.15
Serum cholesterol (mg/dL)	282.80 ± 10.45	250.19 ± 15.66	284.73 ± 22.06	258.05 ± 16.17	0.77	0.09	0.86

Data are presented as means ± SEM, n = 10-11 for each group.

[1]Two-way ANOVA was used to determine the significant effect of fat amount and oil type.

SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard

3. Expression of genes involved in appetite control

The mRNA expression of *Ghsr*, the ghrelin receptor, was significantly higher in control diet-fed mice (P = 0.03) and in PNO-fed mice (P = 0.03). *Ghsr* mRNA level of the PC group was 1.23-fold greater (P = 0.02) than that of the SC group. *Ghsr* mRNA expression was negatively correlated with body weight at 12wk (r = - 0.47, P = 0.03) and the amount of white adipose tissue (r = - 0.46, P = 0.03). On the other hand, neither the fat amount nor the oil type influenced mRNA levels of a major satiety hormone, *Cck*, and its receptors, *Cckar* and *Cckbr*. The mRNA levels of *Ghrelin*, which promotes food intake; and *Goat*, the Ghrelin activating enzyme, were not affected by fat amount and oil type, as well (**Table 7**). There were no significant correlations between *Ghrelin* and *Npy* or *Agrp* mRNA level although ghrelin is known as to promote the expression of NPY and AgRP.

The mRNA level of *Agrp*, one of the neuropeptides that promote appetite and reduce energy expenditure, was significantly higher in PNO-fed mice (P = 0.02). The PHF group had significantly higher *Agrp* expression (2.16-fold, P = 0.02) than the SHF group. However, the mRNA level of *Npy*, another neuropeptide that acts in similar way as *Agrp*, was not significantly influenced by fat amount and oil type. The mRNA levels of *Pomc* and *Cart*, neuropeptides that suppress appetite and increase energy expenditure, tended to be greater in PNO-fed mice (*Pomc*, P = 0.08; *Cart*, P = 0.06) (**Figure 6**). While *Npy* and *Agrp* mRNA levels were negatively correlated

with the amount of white adipose tissue (*Npy*, r = - 0.42, *P* = 0.03; *Agrp*, r = - 0.44, *P* = 0.03), *Pomc* or *Cart* mRNA level had no correlation with it.

Table 7. The mRNA expression levels of cholecystokinin related and ghrelin related genes[1]

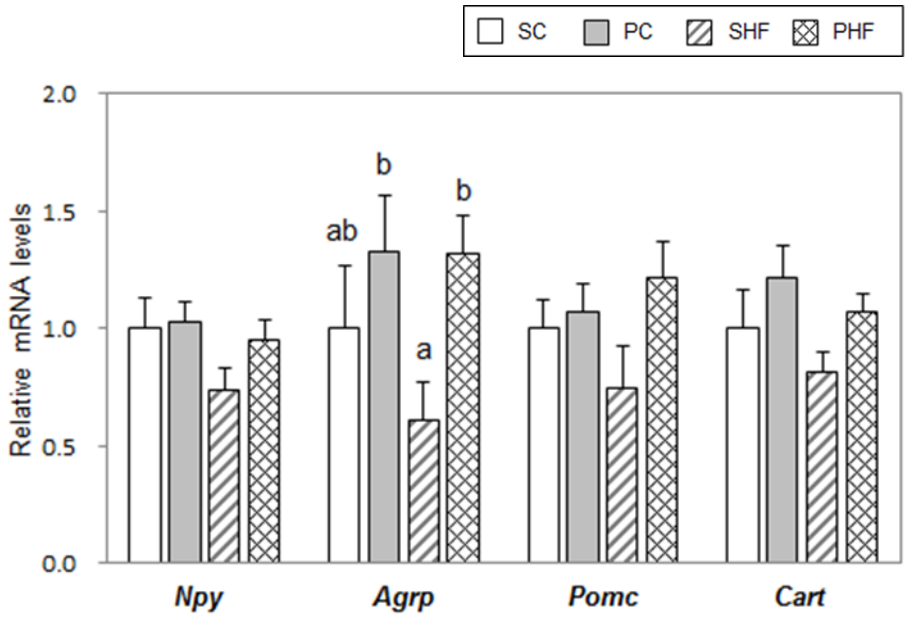
	Control		High-fat		Fat amount (*P*-value)	Oil type (*P*-value)	Inter-action (*P*-value)
	SC	PC	SHF	PHF			
Cck	1.00 ± 0.26	0.99 ± 0.13	0.94 ± 0.15	1.04 ± 0.18	0.98	0.79	0.75
Cckar	1.00 ± 0.28	1.14 ± 0.64	0.43 ± 0.15	0.83 ± 0.32	0.29	0.51	0.74
Ccrbr	1.00 ± 0.16	0.99 ± 0.17	0.93 ± 0.08	0.99 ± 0.19	0.90	0.86	0.75
Ghrelin	1.00 ± 0.09	1.13 ± 0.15	1.02 ± 0.05	0.97 ± 0.18	0.60	0.75	0.51
Goat	1.00 ± 0.11	1.22 ± 0.08	1.08 ± 0.09	0.91 ± 0.14	0.29	0.81	0.09
Ghsr	1.00 ± 0.06[a]	1.23 ± 0.09[b]	0.92 ± 0.06[a]	1.00 ± 0.05[a]	0.03	0.03	0.27

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.

[1]Two-way ANOVA was used to determine the significant effect of fat amount and oil type. Different superscripts indicate significant differences at $P < 0.05$ by Fisher's LSD multiple comparison test.

SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard

Cck, Cholecystokinin; *Cckar*, Cholecystokinin A receptor; *Ccrbr*, Cholecystokinin B receptor; *Goat*, Ghrelin O-acyltransferase; *Ghsr*, Growth hormone secretagogue receptor



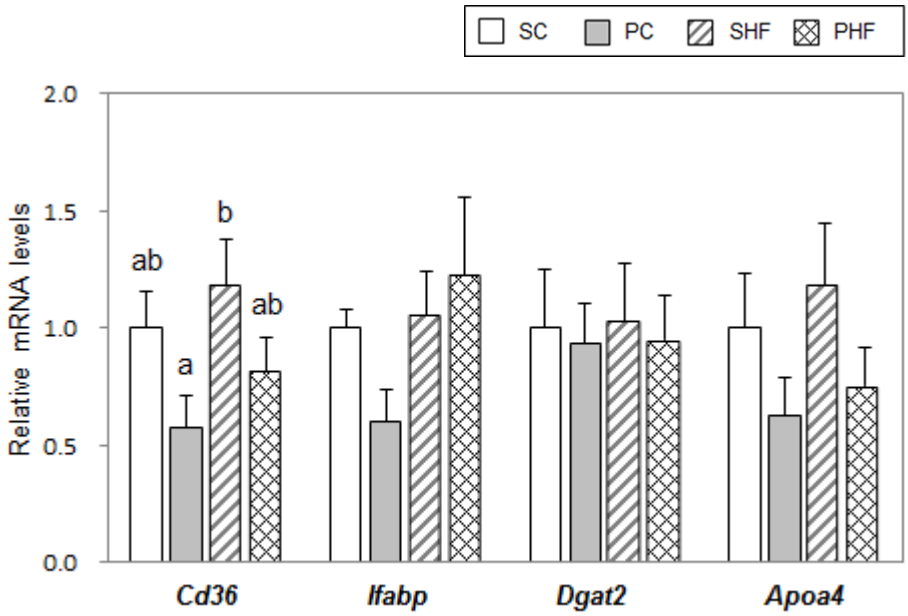
Fat amount (*P*-value)	0.10	0.36	0.71	0.18
Oil type (*P*-value)	0.22	0.02	0.08	0.06
Interaction (*P*-value)	0.35	0.37	0.19	0.87

Figure 6. The mRNA expression levels of neuropeptides (hypothalamic *Npy*, *Agrp*, *Pomc*, and *Cart*). Data are presented as means ± SEM, n= 5-6 for each group. Two-way ANOVA 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. SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard. *Npy*, neuropeptide Y; *Agrp*, Agouti-related peptide; *Pomc*, Pro-opiomelanocortin; *Cart*, Cocaine- and amphetamine-regulated transcript.

4. Expression of genes involved in lipid absorption

In order to investigate whether less body weight and less white adipose tissue of PNO-fed mice were due to alteration in intestinal fat absorption, the mRNA levels of genes involved in intestinal lipid metabolism were determined (**Figure 7**).

PNO-fed mice had significantly lower mRNA level of *Cd36* which transports fatty acids from lumen to enterocytes ($P = 0.03$). PC group had a tendency of lower *Cd36* expression (0.57-fold, $P = 0.09$) than SC group. PNO-fed mice tended to have lower mRNA level of *Apoa4* which facilitates intestinal lipoprotein production ($P = 0.07$). No effects of fat amount and oil type on the mRNA levels of *Ifabp* which transports and metabolizes fatty acids in enterocytes; and *Dgat2* which synthesizes TG from DG were observed.



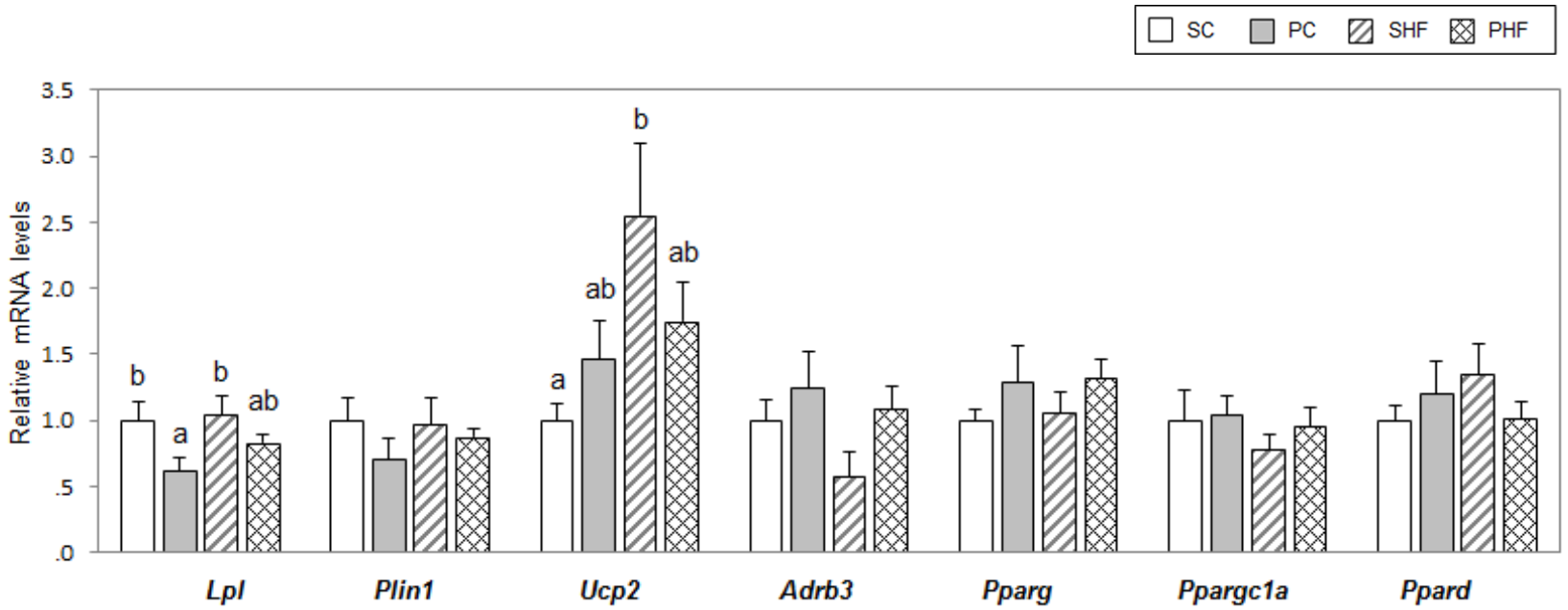
Fat amount (*P*-value)	0.20	0.13	0.92	0.47
Oil type (*P*-value)	0.03	0.60	0.74	0.07
Interaction (*P*-value)	0.85	0.21	0.96	0.88

Figure 7. The mRNA expression levels of genes associated with intestinal lipid metabolism (jejunal *Cd36, Ifabp, Dgat2,* and *Apoa4*). Data are presented as means ± SEM, n= 5-6 for each group. Two-way ANOVA 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. SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard. *Cd36*, Cluster of differentiation 36; *Ifabp*, Intestinal fatty acid binding protein; *Dgat2*, Diacylglycerol O-acyltransferase 2; *Apoa4*, Apolipoprotein A-IV.

5. Expression of genes involved in body fat accumulation

We also examined whether PNO had antiadiposity effect by reducing lipogenesis or by enhancing lipolysis and thermogenesis.

PNO-fed mice had significantly lower mRNA expression of *Lpl* (P = 0.02) which hydrolyzes TG within lipoproteins to diacylglycerol and fatty acids for fatty acid uptake by adipocytes. *Lpl* mRNA level in the PC group was 38% less than SC group (P = 0.04). The mRNA level of *Adrb3*, which enhances lipolysis in white adipose tissue, tended to be higher in PNO-fed mice (P = 0.08). The mRNA level of *Ucp2*, which plays a role in thermogenesis, was significantly higher in HFD-fed mice (P = 0.02). The mRNA levels of *Plin1*, *Pparg*, *Ppargc1a*, and *Ppard* were unaffected by fat amount and oil type (**Figure 8**).



Fat amount (*P*-value)	0.31	0.64	0.02	0.16	0.81	0.35	0.72
Oil type (*P*-value)	0.02	0.21	0.64	0.08	0.16	0.51	0.74
Interaction (*P*-value)	0.46	0.51	0.10	0.54	0.95	0.67	0.19

Figure 8. 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 ± SEM, n=5-6 for each group. Two-way ANOVA

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. SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 10% soybean oil + 35% lard; PHF, 10% pine nut oil + 35% lard. *Lpl*, Lipoprotein lipase; *Plin1*, Perilipin 1; *Ucp2*, Mitochondrial uncoupling protein 2; *Adrb3*, Beta-3 adrenergic receptor; *Pparg*, Peroxisome proliferater-activated receptor gamma; *Ppargc1a*, PPAR gamma coactivator 1 alpha; *Ppard*, Peroxisome proliferator-activated receptor delta.

V. DISCUSSION

In the present study, the replacement of 10% kcal fat with pine nut oil (PNO) in place of soybean oil (SBO) in control diet or in high-fat diet resulted in lower body weight gain and less amount of white adipose tissue. The differences in fat mass between SBO- and PNO-fed mice seemed to be associated with the effect of PNO on lipid metabolism, not with the appetite suppressing effect of PNO because no difference in food intake between SBO and PNO groups was observed. The appetite suppressing effect of PNO is controversial since previous studies showed that PNO significantly reduced appetite (Pasman et al. 2008) or food intake (Hughes et al. 2008), but a recent study showed that PNO did not suppress appetite and energy intake (Verhoef et al. 2011).

We did not observe differences in the mRNA levels of satiety peptides, such as *Cck*, *Cckar*, and *Cckbr*, among groups even though pine nut oil was reported to promote CCK release in STC-1 cell and in post-menopausal overweight women (Pasman et al. 2008). It is possible that 12-hour fasting of mice masked the satiating effect of PNO since CCK signals are induced when food is present in the gut lumen. Further study using refeeding condition is required to examine the effect of PNO on the expression of CCK related genes.

The mRNA expression levels of *Ghrelin*, the major appetite-stimulating

hormone; and *Goat*, Ghrelin activating enzyme, were not different among all groups. This result is in accordance with earlier studies which showed that *Ghrelin* and *Goat* expression were not different between diet-induced obese mice and lean mice after fasting (Moesgaard et al. 2004; Morash et al. 2010). The mRNA expression of *Ghsr* was significantly higher in PC group than other groups, which indicated that mice in PC group received the stronger signal that promotes energy consumption than other groups. GHSR is upregulated when more energy is needed to increase appetite and decrease energy expenditure (Holst et al. 2004). Therefore, PC group, which had the least stored energy among all groups, might have expressed more *Ghsr* mRNA in order to restore its energy storage. *Ghsr* mRNA expression was negatively correlated with body weight at 12wk and the amount of white adipose tissue, whereas *Ghrelin* and *Goat* mRNA expression levels did not correlate with them. It is thought that dietary effect on the mRNA expression of hormone receptors remains for a long-term, whereas that of gastrointestinal hormones and related enzymes does not.

Since we observed the lower fat mass in PNO-fed mice, we expected that the mRNA expression of *Npy* and *Agrp*, which reduce energy expenditure, would be lower; and the mRNA expression of *Pomc* and *Cart*, which increase energy expenditure, would be higher in PNO-fed mice. However, contrary to our expectations, *Agrp* mRNA expression was significantly lower in SHF group compared to PHF group. The lower *Agrp*

mRNA expression in SHF group could be a compensatory mechanism to regulate food intake and energy homeostasis since the mice in SHF group had higher body weight and more amount of white adipose tissue than those in PHF group. This explanation is supported by the fact that the mRNA expression levels of *Npy* and *Agrp* were negatively correlated with body weight at 12wk and white adipose tissue.

Collectively, *Pomc* and *Cart* mRNA expression tended to be greater in PNO-fed mice. The tendency seemed to be mainly due to the low expression of *Pomc* and *Cart* in SHF group. It is possible that mice in SHF group failed to increase *Pomc* and *Cart* mRNA expression despite their higher white adipose tissue weight and leptin level. Leptin stimulates *Pomc* and *Cart* mRNA expression and inhibits *Npy* and *Agrp* mRNA expression to maintain energy homeostasis by decreasing food intake and weight gain (Badman et al. 2005). Therefore, low mRNA expression of *Pomc* and *Cart* in SHF group suggests that certain degree of leptin resistance was present in SHF group, which might cause the POMC/CART pathway dysregulation. The lower fat mass of PHF group might have prevented the development of leptin resistance.

Lin et al. (2000) reported that POMC/CART pathway was damaged at earlier stage of high-fat diet-induced obesity compared with the NPY/AgRP pathway, which are similar to the results of the present study. In a study by Lin et al. (2000), 8 week high-fat diet feeding did not lead to upregulation of

Pomc mRNA expression. Rather, after 19 weeks of high-fat diet feeding, *Pomc* mRNA expression was downregulated. On the contrary, *Npy* mRNA expression was downregulated properly by high-fat feeding at both 8 and 19 weeks of time point.

The differences in the expression of the appetite controlling genes are considered to be caused by the differences in body weight and the amount of white adipose tissue. Since there was no significant difference in food intake between SBO- and PNO-fed mice, we speculated that other effects of PNO, not satiating effect, have contributed to the lower weight gain and less amount of white adipose tissue. Therefore, we examined whether PNO replacement altered lipid metabolism in intestine and white adipose tissue.

To investigate the effect of PNO on lipid absorption in small intestine, jejunal *Cd36*, *Ifabp*, *Dgat2*, and *Apoa4* mRNA expression were determined. CD36, which is a membrane protein, enhances fatty acids absorption in small intestine and peripheral utilization (Drover et al. 2005). ApoA-IV, which regulates chylomicron assembly, increases the efficiency of intestinal lipid absorption and it eventually facilitates weight gain and adipose tissue lipid storage (Simon et al. 2011). IFABP targets dietary fatty acids for TG synthesis (Lagakos et al. 2011), and DGAT2 catalyzes the final step in TG synthesis (Abumrad et al. 2012). Mice fed chronic high-fat diet were reported to adapt to the fat content of the diet by increasing mRNA expression of these genes (Petit et al. 2007; Uchida et al. 2012).

In the present study, PNO-fed mice had significantly lower *CD36* mRNA expression and tended to have lower *Apoa4* mRNA expression than SBO-fed mice. These results suggest that dietary lipids may not have been efficiently absorbed with inclusion of PNO in the diet. This may have led to the lower fat mass and body mass in PNO-fed mice. In other study with similar study design, non-esterified fatty acid (NEFA) levels in feces of PNO-fed mice were significantly higher than those of SBO-fed mice (P = 0.04). Fecal NEFA excretion in PHF group was significantly higher than those in SHF group (37% higher, $P < 0.01$) (**Appendix 3**). This data supports the idea that PNO is less efficiently absorbed than SBO. No differences in the mRNA expression of *Ifabp* and *Dgat2* were detected among groups. These results indicated that PNO did not affect TG synthesis from dietary fatty acids.

To investigate the effect of PNO on body fat accumulation in white adipose tissue, epididymal *Lpl*, *Plin1*, *Ucp2*, *Adrb3*, *Pparg*, *Ppargc1a*, and *Ppard* mRNA expression were determined. Lipoprotein lipase promotes influx of TG from lipoprotein to adipocyte (Mead et al. 2002). Perilipin 1 coats lipid droplets to prevent lipid mobilization (Large et al. 2004). Uncoupling protein 2 regulates adaptive thermogenesis (Diano et al. 2011), and β3-adrenergic receptor enhances hydrolysis of stored energy in white adipocyte (Collins et al. 2010). PPARγ regulates the formation of fat cells and their ability to store lipids. PGC-1α induces the expression of genes

essential for mitochondrial biogenesis. PPARδ stimulates fat-burning by inducing the expression of genes involved in fatty acid oxidation and thermogenesis to prevent obesity (Evans et al. 2004). It is reported that high-fat diet-induced obesity could be alleviated by decreasing *Lpl*, *Plin1*, and *Pparg* mRNA expression and increasing *Ucp2*, *Adrb3*, and *Ppargc1a* mRNA expression (Lee et al. 2011; Chen et al. 2012).

In PNO-fed mice, *Lpl* mRNA expression was significantly lower, and *Adrb3* mRNA expression tended to be higher compared with the SBO-fed mice. These results indicate that PNO has the potential for attenuating the body fat accumulation. PNO does not seem to affect thermogenesis since *Ucp2* mRNA expression was affected by fat amount, but not by oil type. Higher expression of *Ucp2* mRNA expression in high-fat diet-fed mice was also observed by others, and this was considered as a defense mechanism against diet-induced obesity (Rippe et al. 2000). The mRNA expression levels of *Plin1*, *Pparg*, *Ppargc1a*, and *Ppard* were not significantly different among groups.

In conclusion, PNO-enriched diet feeding reduced weight gain in high-fat diet induced obese mice and the amount of white adipose tissue in both control diet- and high-fat diet-fed mice. The low mRNA expression levels of *Pomc* and *Cart* in SHF group suggest the possibility of POMC/CART pathway dysregulation in SHF group, which was not observed in PHF group. The lower *Cd36* mRNA expression and the tendency of lower *ApoA4*

mRNA expression in PNO-fed mice indicate that PNO has the lower efficiency of absorption in small intestine. The lower *Lpl* mRNA expression and the tendency of higher *Adrb3* mRNA expression suggest that PNO is less efficiently stored in white adipose tissue. These characteristics of PNO seemed to contribute to a less accumulation of fat mass in PNO-fed mice.

VI. SUMMARY

In this study, the effects of Korean pine nut oil (PNO) compared with soybean oil (SBO) on the factors involved in body fat accumulation were investigated. After feeding mice for 12 weeks with control diets containing 10% kcal fat from PNO or SBO (PC or SC) or high-fat diets containing 35% kcal fat from lard and 10% kcal fat from PNO or SBO (PHF or SHF), body weight, food intake, the amount of white adipose tissue, serum leptin, triglyceride, and cholesterol levels, and the expression of genes involved in appetite control and lipid metabolism were measured. The results of the present study were as follows:

1) PNO-fed mice had significantly lower body weight at 12wk and weight gain than SBO-fed mice although there was no difference of daily food intake between SBO- and PNO-fed mice. PHF group had significantly lower body weight at 12wk and weight gain than SHF group.
2) PNO-fed mice had significantly less white adipose tissue and serum leptin level than SBO-fed mice. PC group had significantly less white adipose tissue than SC group. PHF group had significantly less white adipose tissue and serum leptin level than SHF group.
3) No significant difference in serum TG level was detected while PNO-fed mice had the tendency of lower serum cholesterol level than

SBO-fed mice.

4) PNO-fed mice had significantly higher *Ghsr* and *Agrp* mRNA expression and tendency of higher *Pomc* and *Cart* mRNA expression than SBO-fed mice in hypothalamus. PC group had significantly higher *Ghsr* mRNA expression than SC group, and PHF group had significantly higher *Agrp* mRNA expression than SHF group.
5) PNO-fed mice had significantly lower *Cd36* mRNA expression and had the tendency of lower *Apoa4* mRNA expression in small intestine.
6) PNO-fed mice had significantly lower *Lpl* mRNA expression and the tendency of higher *Adrb3* mRNA expression in white adipose tissue. PC group had significantly lower *Lpl* mRNA expression than SC group.

These results indicate that PNO-enriched diet feeding reduced weight gain and the amount of white adipose tissue. The low mRNA expression levels of *Pomc* and *Cart* in SHF group suggest the possibility of POMC/CART pathway dysregulation in SHF group, which was not observed in PHF group. PNO has the lower efficiency of absorption and storage in the body. These characteristics of PNO seemed to contribute to a less accumulation of fat mass in PNO-fed mice.

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APPENDICES

Appendix 1. Determination of fecal lipid concentrations

In other study with similar study design, feces were collected for 3 days during the 12th week of feeding the experimental diets and freeze-dried for 72 hours. Fecal lipids were extracted using a Folch extraction protocol (Folch et al. 1957). Briefly, 50mg of freeze-dried feces was homogenized in 2 mL of chloroform and 1 mL of methanol. After 1 mL of distilled water was added, the homogenized samples were shaken by a see-saw rocker for 20 minutes at room temperature and centrifuged at 3000 rpm for 20 minutes at room temperature. The lower phase of each sample was transferred to the fresh tubes and dried overnight. The dried lipid samples were redissolved in 1 mL of isopropanol to determine the concentrations of triglyceride (TG), non-esterified fatty acids (NEFA), and cholesterol.

Fecal TG concentration was determined using commercial kit (Asan Pharmaceutical, Korea) based on enzymatic assay. The enzyme mixture in the solution hydrolyzes TG to glycerol and fatty acids, phosphorylates glycerol into glycerophosphoric acid, and oxidizes glycerophosphoric acid. Oxidation of glycerophosphoric acid creates hydrogen peroxide which produces quinoid dyes by reacting with 4-aminoantipyrine, N-ethyl-N-sulfopropyl-m-toluidine, and peroxidase. Fecal TG content was calculated on the absorbance of quinoid dyes. In this study, 5 μL of lipid samples or

standard (300 mg/dL of glycerol) and 300 μL of the enzyme solution were added to each well of 96-well plates and incubated for 10 minutes at 37°C. The absorbance was measured using a microplate reader (Spectramax 190, Molecular Devices, CA, USA) set to 550nm.

Fecal NEFA concentration was determined using commercial kit (Shinyang Diagnostics, Korea) based on enzymatic assay. The enzyme mixture in the solution converts NEFA to Acyl-CoA and oxidizes Acyl-CoA into 2,3-trans-enoyl-CoA and hydrogen peroxide. The hydrogen peroxide creates quinone dyes by reacting with 4-aminoantipyrine, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine, and peroxidase. The contents of NEFA in feces are calculated on the absorbance of quinone dyes. In this study, 5 μL of lipid samples or standard (1 mEq/L of oleic acid) and 200 μL of the enzyme solution-1 were added to each well of 96-well plates and incubated for 10 minutes at 37°C. After incubation, 100 μL of the enzyme solution-2 was added and incubated for 10 minutes at 37°C. The absorbance was measured using an identical microplate reader used in quantification of fecal TG concentration set to 546nm and 600nm. Fecal NEFA content was calculated by subtracting the absorbance at 600nm from that at 546nm.

Fecal cholesterol concentration was determined using commercial kit (Asan Pharmaceutical, Korea) based on enzymatic assay. The enzyme mixture in the solution hydrolyzes esterified cholesterol to free cholesterol and fatty acids, and oxidizes free cholesterol into Δ^4-cholestenone and

hydrogen peroxide. The hydrogen peroxide creates quinone dyes by reacting with 4-aminoantipyrine, phenol, and peroxidase. Fecal cholesterol content was calculated on the absorbance of quinone dyes. In this study, 5 μL of lipid samples or standard (300 mg/dL of esterified cholesterol) and 300uL of enzyme solution were added to each well of 96-well plates and incubated for 5 minutes at 37°C. The absorbance was measured using an identical microplate reader used in quantification of fecal TG concentration set to 500nm.

Appendix 2. Body weight, weight gain, food intake, and feces of the mice[1]

	Control		High-fat		Fat amount (*P*-value)	Oil Type (*P*-value)	Inter-action (*P*-value)
	SC	PC	SHF	PHF			
Body weight at 0 wk (g)	16.85 ± 0.23	17.04 ± 0.35	16.90 ± 0.22	16.92 ± 0.21	0.89	0.68	0.73
Body weight at 12 wk (g)	32.69 ± 0.70[a]	32.82 ± 0.88[a]	43.35 ± 0.91[b]	42.53 ± 0.77[b]	< 0.01	0.70	0.59
Body weight gain (g)	15.83 ± 0.81[a]	15.78 ± 0.82[a]	26.45 ± 1.01[b]	25.61 ± 0.71[b]	< 0.01	0.64	0.68
Daily food intake (g)	3.37 ± 0.04[b]	3.37 ± 0.06[b]	3.01 ± 0.03[a]	2.98 ± 0.04[a]	< 0.01	0.68	0.63
Daily energy intake (kcal)	12.95 ± 0.14[a]	12.97 ± 0.23[a]	14.25 ± 0.15[b]	14.09 ± 0.18[b]	< 0.01	0.67	0.61
Feces[2] (g/day)	0.27 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.16	0.56	0.35
Freeze-dried feces[3] (g/day)	0.23 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	0.04	0.72	0.45

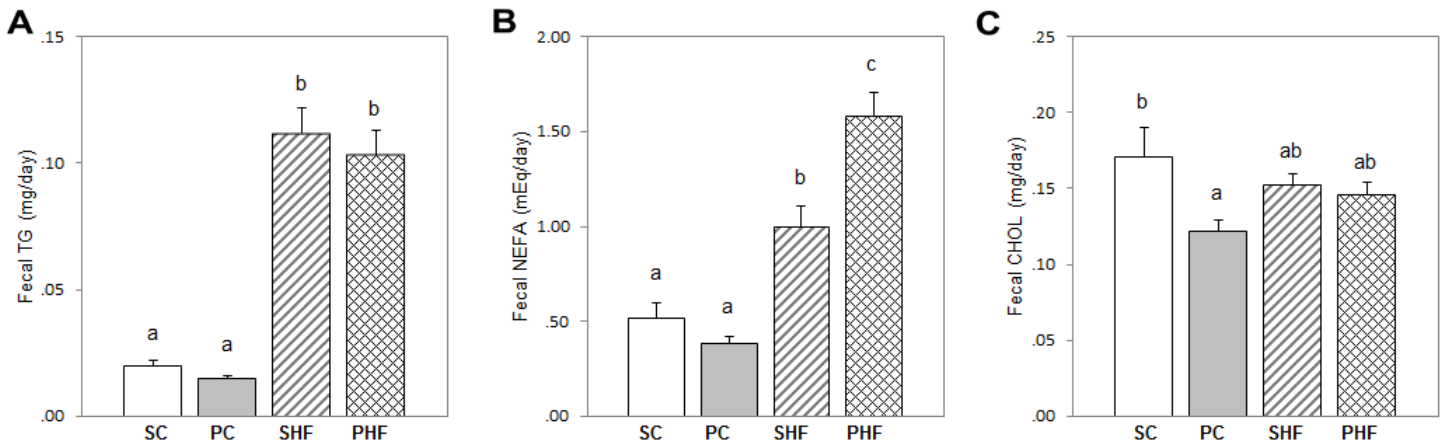
Data are presented as means ± SEM.

[1]Two-way ANOVA was used to determine the significant effect of fat amount and oil type. Different superscripts indicate significant differences at $P < 0.05$ by Fisher's LSD multiple comparison test.

[2]Feces were collected for 3 days during the 12th week of feeding the experimental diets.

[3]Feces were freeze-dried for 72 hours.

SC, 10% soybean oil, n=14; PC, 10% pine nut oil, n=7; SHF, 10% soybean oil + 35% lard, n=14; PHF, 10% pine nut oil + 35% lard, n=10.



Fat amount (*P*-value)	< 0.01
Oil type (*P*-value)	0.41
Interaction (*P*-value)	0.84

Fat amount (*P*-value)	< 0.01
Oil type (*P*-value)	0.04
Interaction (*P*-value)	< 0.01

Fat amount (*P*-value)	0.84
Oil type (*P*-value)	0.06
Interaction (*P*-value)	0.14

Appendix 3. Fecal TG, NEFA, and CHOL levels. A, Fecal triglyceride level. B, Fecal non-esterified fatty acid level. C. Fecal total cholesterol level. Data are presented as means ± SEM. Feces were collected for 3 days during the 12th week of feeding the experimental diets, and fecal lipid contents were measured by enzymatic assay. Two-way ANOVA 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, n=14; PC, 10% pine nut oil, n=7; SHF, 10% soybean oil + 35% lard, n=14; PHF, 10% pine nut oil + 35% lard, n=10.

국문초록

고지방 식이로 유도된 비만 마우스에서 잣기름이 체지방량 조절 관련 요인에 미치는 영향

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잣기름은 소화관 호르몬 분비에 영향을 주어 식욕을 줄이고 식이섭취량을 감소시킨다고 보고되어 왔다. 그러나 잣기름이 소화관 호르몬 수용체나 신경펩타이드에 미치는 영향에 대해서는 연구된 바가 없으며, 잣기름이 지질 대사에 미치는 영향에 대한 연구도 부족한 실정이다. 본 연구에서는 잣기름이 체지방량 조절 관련 요인에 미치는 영향을 알아보고자 하였다. 5 주령의 수컷 C57BL/6 mice 를 네 군으로 나눈 후 네 가지 실험식이를 각각 12 주간 제공하였다. 실험 식이는 총 식이 칼로리의 10%를 콩기름이나 잣기름으로 공급하는 저지방 식이(SC 또는 PC)와 총 식이 칼로리의 45% 중 35%는 라아드로, 10%는 콩기름이나 잣기름으로 공급하는 고지방 식이(SHF 또는 PHF)였다. Cholecystokinin 관련 유전자, Ghrelin 관련 유전자, 신경펩타이드, 그리고 소장 및 백색 지방에서

의 지질 대사 관련 유전자의 mRNA 발현량을 Real-time PCR 로 측정하였다. 전체적으로 잣기름 섭취군에서 체중 증가량(P = 0.01)과 백색 지방량(P < 0.01)이 적었으나, 콩기름 섭취군과 잣기름 섭취군의 식이 섭취량에는 차이가 없었다. PC 군과 PHF 군의 백색 지방량은 각각 SC 군 (30% 적음, P = 0.05)과 SHF 군의 백색 지방량(18% 적음, P = 0.03) 보다 적었다. 잣기름 섭취군의 시상하부에서 *Growth hormone secretagogue receptor* 및 *Agouti-related protein* 의 발현량은 유의적으로 높았고 (*Ghsr,* P = 0.03; *Agrp,* P = 0.02), *Pro-opiomelanocortin* 과 *Cocaine- and amphetamine-regulated trascript* 의 발현량도 높은 경향성을 보였다 (*Pomc,* P = 0.08; *Cart,* P = 0.06). PC 군은 SC 군에 비해 더 높은 *Ghsr* 발현량을 보였고 (1.23 배, P = 0.02), PHF 군은 SHF 군에 비해 더 높은 *Agrp* 발현량을 보였다 (2.16 배, P = 0.02). 공장에서의 *Cd36* 과 *Lipoprotein lipase* 의 발현량은 잣기름 섭취군에서 유의적으로 낮았으며 (*Cd36,* P = 0.03; *Lpl,* P = 0.02), PC 군은 SC 군에 비해 더 낮은 *Lpl* 발현량을 보였다 (38% 적음, P = 0.04). 잣기름 섭취군은 또한 콩기름 섭취군과 비교했을 때, 낮은 경향성의 공장 *Apolipoprotein A-IV* 발현량 (P = 0.07)과 높은 경향성의 공장 *β3-adrenergic receptor* 발현량을 보였다 (P = 0.08). 잣기름 섭취군에서 *Ghsr* 과 *Agrp* 의 발현량이 높았던 것은 콩기름 섭취군보다 백색 지방량이 적었던 잣기름 섭취군에서 에너지 섭취를 촉진하는 신호가 더욱 강하게 발생 및 전달되었다는 것을 의미한다. 그리고 잣기

름 섭취군에서 *Pomc* 와 *Cart* 의 발현량이 높은 경향성을 보였던 것은 비만 정도가 더 심했던 SHF 군에서 POMC/CART 신호 전달 경로가 손상되었을 가능성이 있다는 것을 암시한다. 반면, 체중과 체지방량이 적었던 PHF 군에서는 이 POMC/CART 신호 전달 체계가 손상되지 않은 것으로 추측된다. 잣기름 섭취군에서 유의적으로 낮았던 *Cd36* 및 *Lpl* 발현량과, 낮은 경향성의 *Apoa4* 발현량, 그리고 높은 경향성의 *Adrb3* 발현량은 잣기름이 콩기름보다 적게 흡수 및 저장됨을 의미한다. 결론적으로 본 연구는 고지방 식이를 섭취한 마우스에서 잣기름이 체중 증가량과 백색 지방량을 줄임으로써 POMC/CART 신호 전달 체계가 손상될 가능성을 줄일 수 있음을 시사한다. 그리고 잣기름 섭취군의 낮은 백색 지방량은 잣기름이 지질 대사에 미치는 영향에 의한 것이라 사료된다.

주요어: 잣기름, 고지방 식이, POMC/CART 신호 전달 체계, 공장 지질 흡수, 부고환 지방 지질 대사

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