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The Effect of Pine Nut Oil on Intestinal and Hepatic Lipid Metabolism in High-Fat Diet-Induced Obese Mice

고지방 식이로 유도된 비만 마우스에서 잣기름이 소장과 간 지방 대사에 미치는 영향

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Abstract

The Effect of Pine Nut Oil on Intestinal and Hepatic Lipid Metabolism in High-Fat Diet-Induced Obese Mice

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Consumption of pine nut oil (PNO) was shown to reduce weight gain and attenuate hepatic steatosis in mice fed with high fat diet (HFD). In this study, we aimed to explore the effects of PNO on both intestinal and hepatic lipid metabolism in mice fed a HFD. Five-week-old C57BL/6 mice were fed control diets containing 10% energy fat from either soybean Oil (SBO) or PNO (SC or PC groups), or HFD (45% energy from fat) containing 15% energy fat from lard and 30% energy fat from SBO or PNO (SHF or PHF groups) for 12 weeks. Expression of genes related to intestinal fatty acid (FA) uptake and channeling (*Cd36*, *Fatp4*, *Acsl5*, *Acbp*), intestinal chylomicron synthesis (*Mtp*, *ApoB48*, *ApoA4*), hepatic lipid uptake and channeling (*Lrp1*, *Fatp5*, *Acsl1*, *Acbp*), hepatic triacylglycerol (TG) lipolysis and FA oxidation (*Atgl*, *Cpt1a*, *Acadl*, *Ehhadh*, *Acaa1*), as well as very low-density lipo-

protein (VLDL) assembly (*ApoB100*) were determined by real-time PCR. Overall, replacement of SBO with PNO resulted in significantly less body weight gain (P<0.05) and less white adipose tissue weight (P<0.05). Serum non-esterified fatty acid (NEFA) concentration was significantly higher in mice with PNO consumption. In intestine, PNO-fed mice had significantly lower *Cd36* mRNA expression (P<0.05). *ApoA4* mRNA levels were significantly lower in PHF compared with SHF (P<0.05). In addition, PNO consumption tended to result in higher hepatic mRNA levels of *Atgl* (P=0.08) and *Cpt1a* (P=0.05). The mRNA levels of *Acadl* and *ApoB100* were significantly higher in mice fed PNO diet (P<0.05). Together, lower *Cd36* and *ApoA4* mRNA expression in PNO consumption groups suggest that PNO may decrease activities of intestinal FA uptake and chylomicron assembly in intestine. The tendency of higher *Atgl* and *Cpt1a* mRNA expression, together with the significantly higher *Acadl* and *ApoB100* mRNA expression in PNO-fed group may imply that PNO could increase hepatic TG lipolysis; mitochondrial fatty acid oxidation and VLDL assembly. In conclusion, PNO replacement may function to prevent excessive lipid uptake by intestine as well as improve hepatic lipid metabolism in HFD fed mice. Our findings may indicate PNO as potential dietary supplement for preventing metabolic dysregulations of lipids in intestine and liver seen with obesity.

Keywords: Pine nut oil, Intestine, Liver, Lipid metabolism, High-fat diet

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List of Abbreviations

PNO	pine nut oil
HFD	high fat diet
WAT	white adipose tissue
FA	fatty acid
TG	Triacylglycerol
VLDL	very low density lipoprotein
NEFA	non-esterified fatty acids
FATP	fatty acid transporter
CD36	cluster of differentiation 36
ACSL	acyl-CoA synthetase long-chain
ACBP	acyl-CoA-binding protein
MTP	microsomal triglyceride transfer protein
APOB	apolipoprotein B
APOA	apolipoprotein A
LRP	low density lipoprotein receptor-related protein
ATGL	adipose triglyceride lipase
CPT	carnitine palmitoyltransferase
ACADL	long Chain Acyl-CoA Dehydrogenase
EHHADH	enoyl-Coenzyme A, hydratase/ 3-hydroxyacyl Coenzyme A dehydrogenase
ACAA	acetyl-Coenzyme A acyltransferase
DGAT	diacylglycerol O-acyltransferase
LCFA	long chain fatty acid

I. Introduction

A dietary pattern characterized by high fat is considered to be a major contributor to the development of obesity, which is an increased risk factor for many diseases such as dyslipidemia, metabolic syndrome and steatohepatitis (de Wit N et al., 2012). It is well known that small intestine and liver play pivotal roles in the regulation of lipid metabolism (Abumrad and Davidson, 2012; de Wit N et al., 2012; Kim et al., 2004; Nguyen et al., 2008). The small intestine is considered to be the first interface between body and dietary lipid for its essential role on lipid digestion and absorption and determines the amount and type of lipid entering body. Enterocytes on gut can also sense the luminal contents which provokes changes in intestinal metabolism and further lead to more systemic effects by sending signaling molecules such as gut hormones to liver, muscle or brain which can respond to sustain homeostatic control (de Wit N et al., 2012). Liver, as it is a major organ that performs a diverse range of functions necessary for whole-body metabolic homeostasis (Fabbrini et al., 2010), plays an important role in systemic lipid homeostasis through its ability to take in triacylglycerol (TG), synthesis and store fatty acid (FA) as TG and to secret TG in the form of very low-density lipoprotein (VLDL) (Turpin et al., 2011). It has been suggested that the changes in the amount as well as composition of dietary fat contribute to the alterations in hepatic lipid metabolism and hepatic lipid accumulation (Fabbrini et al., 2010; Green and Hodson, 2014).

Korean pine (*Pinus koraiensis*) nuts have been used in Asia for various dishes. The oil extracted from it, also known as Korean pine nut oil, is the only conifer nut oil rich in pinolenic acid ($18:3^{\Delta 5,9,12}$), which is an unsaturated polymethylene-interrupted fatty acid (UPIFA) with a cis-5 ethylenic bond. Besides the high content of Δ-UPIFA (17.7%), of which pinolenic acid (14.9%) is a major component, the other main FA of PNO are linoleic acid ($18:2^{\Delta 9,12}$, 48.4%), and oleic acid ($18:1^{\Delta 9}$, 25.5%) (Lee et al., 2004).

The effects of PNO on weight control and lipid metabolism have been studied for years. Ferramosca et al. (Ferramosca et al., 2008) showed that mice fed with PNO-containing diet resulted in lower body weight gain, liver weights, and total serum cholesterol and TG levels in comparison with mice fed with maize oil-contained diet. Consumption of a diet containing PNO in mice also resulted in lower serum TG and VLDL-TG levels compared with mice fed diets containing other oils such as sunflower or linseed oils (Asset et al., 1999).

Previously studies have compared the effects of PNO with soybean oil (SBO) on the factors involved in body fat accumulation as well as development of hepatic steatosis in HFD induced obese mice. In a study where HFD containing 45% kcal fat from 10% PNO and 35% lard compared with similar HFD with 10% SBO instead, PNO groups had significantly lower body weight and white adipose tissue weight, lower serum cholesterol concentration, higher fecal NEFA content and

lower hepatic TG level, which all indicated that PNO may contribute to less lipid accumulation in HFD fed mice.

High pinolenic acid containing fatty acid extracts has reported to have influence on lipid metabolism such as lowering cholesterol level in comparison to low pinolenic acid contatining fatty acid extract in HepG2 cells (Heath et al., 2003). Higher content of PNO may exert greater influence on health modulation. In a study by Le et al. (Le et al., 2012), mice fed a HFD with 15% energy from lard and 30% energy form either SBO or PNO, enhanced mitochondrial FA oxidative metabolism in skeletal muscle and brown adipose tissue was afterwards observed in obese mice with PNO consumption evidenced by higher expression of genes related to FA oxidation such as *Ppara*, *Cpt1* and *Acadl*.

However, the effects of higher PNO content on intestinal and hepatic lipid metabolism in mice fed with HFD haven't been studied. Therefore the current study aims to investigate the role of higher PNO content in HFD (PNO contributes 30% kcal in a total 45% kcal fat contained HFD) on body weight loss, serum lipid content as well as intestinal and hepatic lipid metabolism.

II. Literature Review

1. Intestinal fatty acid absorption and chylomicron secretion

Over consumption of dietary lipid is generally considered to be the major causative factor of obesity. The absorption of dietary lipid by the small intestine is highly efficient (>95%) and virtually doesn't depend on the amount of fat consumed (Shim et al., 2009). In view of current pandemic of obesity and the increased consumption of fat in average diet, limiting intestinal lipid absorption as a means of reducing calorie intake has appealed considerable attention (Abumrad and Davidson, 2012).

Cellular long-chain fatty acid uptake

Long chain fatty acid (LCFA), which is generated by lingual and pancreatic lipases from dietary triglycerides, is predominantly absorbed in jejunum (Stahl et al., 2001). There are two mechanisms that have been suggested for the absorption of fatty acid by small intestinal cells: a protein-independent passive diffusion model and a protein-dependent active transport model, which uses fatty acid transport protein (FATP) (Iqbal and Hussain, 2009). Among all proteins that have been proposed to take part in intestinal lipid absorption mechanisms, cluster of differentiation (CD36) and FATP4 are well presented to be the main uptake proteins. Besides the effective role in facilitating tissue FA uptake, CD36, which is highly expressed in

the intestine on the apical side of enterocytes and on endothelial and immune cells throughout the organ, is also reported to be involved in directing the FA to chylomicron formation (Nassir et al., 2007). For FATP4, as its special location is near to endoplasmic reticulum (ER) and subapical membranes, it not only strictly participates in FA uptake process, but also has endogenous acyl-CoA synthetase (ACS) activities (Abumrad and Davidson, 2012). It is reported that animals which experience long episodes of fasting regulate digestive performance widely with feeding and fasting while animals that feed relatively frequently, short term fasting (less than 2 days) doesn't exhibit big changes in absorptive performance (Secor, 2005). Fasting process in our study is regarded to exert little influence on intestinal absorptive capacity.

Cellular Long-chain fatty acid channeling

Once entering the enterocyte, intracellular LCFAs are rapidly be coupled to coenzyme A (CoA) by long-chain fatty acyl-CoA synthetases (ACSLs), stopping their flowing out, with Acyl-CoA binding proteins (ACBPs) functioning to incorporate CoA to LCFA forming LCFA-CoA esters. (Stahl et al., 2001) Intestine-specific ACSL5 functions to catalyze the metabolism of exogenous fatty acid, to participate in the absorption of dietary LCFA as well as to partition FA toward TG synthesis (Yan et al., 2015). In addition, ACBP plays an important role in transporting FA-CoAs to different organelles, ER or nucleus for instance. It might facilitate FA-CoA

desorption from Fatp4 and Acsl5, and thus contribute to the lipoprotein synthesis (Niot et al., 2009).

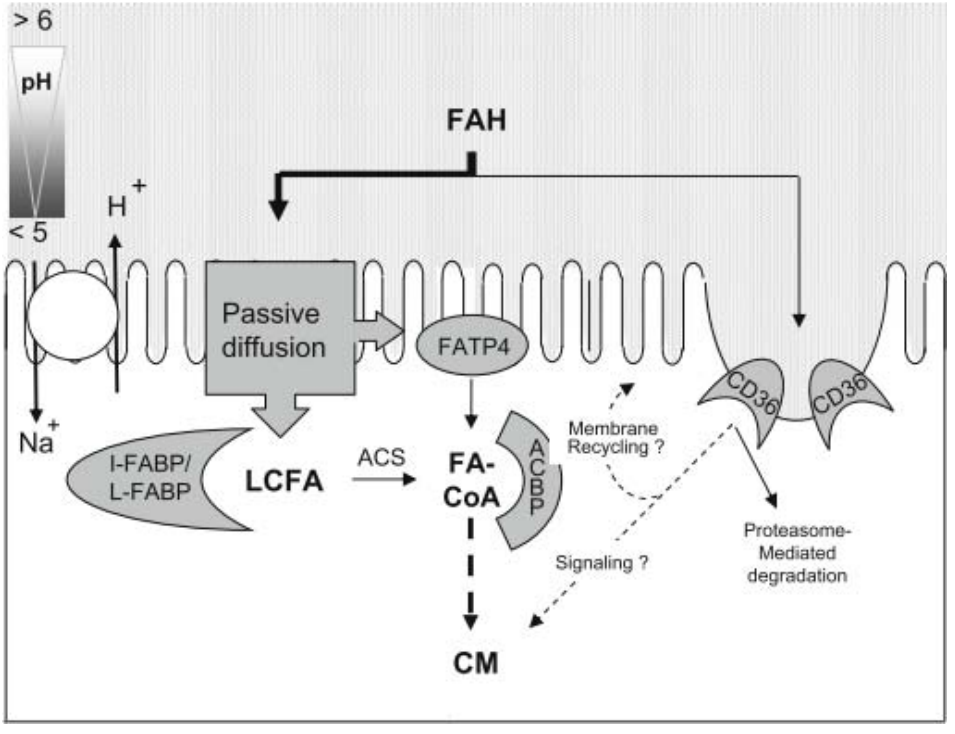


Figure 1. Intestinal absorption of long-chain fatty acids: a working model[1] (Niot et al., 2009).

[1]ACBP, acyl-CoA-binding proteins; CD36, cluster of differentiation; ACS, acyl-CoA synthetases; CM, chylomicrons; FA-CoA, long-chain acyl-CoA; FAH, protonated long-chain fatty acids; FATP4, fatty acid transport protein 4; I-FABP, intestinal fatty acid-binding protein; LCFA, long-chain fatty acids; L-FABP, liver fatty acid-binding protein.

Chylomicron assembly and secretion

Lipids that have been absorbed into the ER are resynthesized and packaged into chylomicrons. The surface structural protein, apolipoprotein B48 (ApoB48) is necessary for chylomicron assembly which also accelerate the formation of lipoprotein on the ER. Microsomal triglyceride transferase (MTP) supports chylomicron biogenesis, functioning as a transporter to shuttle neutral lipid from ER to the acceptor ApoB48 molecule (Abumrad and Davidson, 2012). In this way, MTP lapidates the newly channeled ApoB-48 to form a primordial chylomicron, which is further lipidated with the addition of core TG and cholesteryl ester by MTP to form a pre-chylomicron. Another surface structural protein apolipoprotein A4 (ApoA4), functions as a stabilizer on chylomicron to withhold the chylomicron within the ER, allowing the formation of a larger particle by reloading additional core lipidation (Black, 2007). Pre-chylomicrons are transferred from ER to the Golgi apparatus in which the final maturation of chylomicron takes place. Chylomicrons move from the intestinal mucosa into the lymphatic system, and further enter the blood.

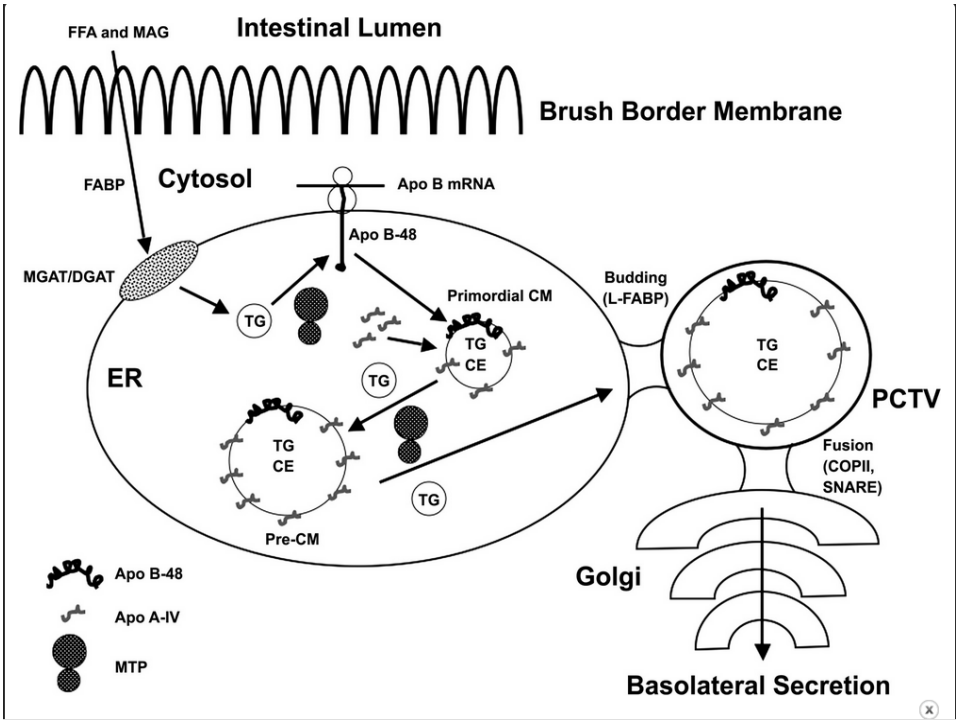


Figure 2. Enterocyte chylomicron assembly and trafficking[1] (Black, 2007).

[1]FFA, free fatty acids; MAG, mono-acylglycerides; ER, endoplasmic reticulum; FABP, fatty acid binding proteins; TG, triacylglycerol; MGAT, acyl-CoA:monoacylglycerol acyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; ApoB48, translated apolipoprotein B-48; MTP, microsomal triglyceride transferase; CE, cholesteryl; Apo A-IV, translated apolipoprotein A-IV; PCTV, pre-CM transport vesicle; COPII, coating protein II; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

2. Hepatic lipid uptake

The liver plays a unique, central role in regulating lipid metabolism. However outpouring lipid intake or any changes in specific pathways of FA metabolism could significantly influence the hepatic function and diseases. Besides, it could also interfere the metabolism of other nutrients, cause extra-hepatic physiology, and even lead to the development of metabolic diseases (Mashek, 2013). It is reported that liver lipid accumulation happens when the amount of TG resulted from hepatic FA and chylomicron remnant taken from plasma and de novo lipogenesis overwhelms the amount of TG used for lipolysis and FA oxidation as well as for export with VLDL (Fabbrini et al., 2010).

Hepatic chylomicron remnant clearance

Chylomicrons are formed in the intestine and transport dietary TG to peripheral tissues. On the way to liver, plasma lipoprotein lipase hydrolyzes chylomicron TG allowing the delivery of free FA to muscle and adipose tissue. As a result, a new particle called a chylomicron remnant is formed. It is reported that excessive chylomicron remnants concentration in plasma is a risk factor for the development of cardiovascular disease whereas liver plays an important role on rapid chylomicron remnant clearance. Besides this hepatic clearance can be down-regulated by high fat diet (HFD) (Mortimer et al., 1995).

The lipolysis-produced CR can be rapidly cleared by liver via an apolipoprotein E (ApoE)-mediated process. There are two candidates for a remnant receptor on liver, low density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP) but LRP is verified to particularly have high affinity of ApoE binding (Beisiegel et al., 1991; Cooper, 1997). In addition, both lipoprotein lipase and hepatic lipase, which are involved in the uptake and metabolism of chylomicron in liver, have been described as binding to LRP to mediate the transferring lipoprotein into cells (Krapp et al., 1996).

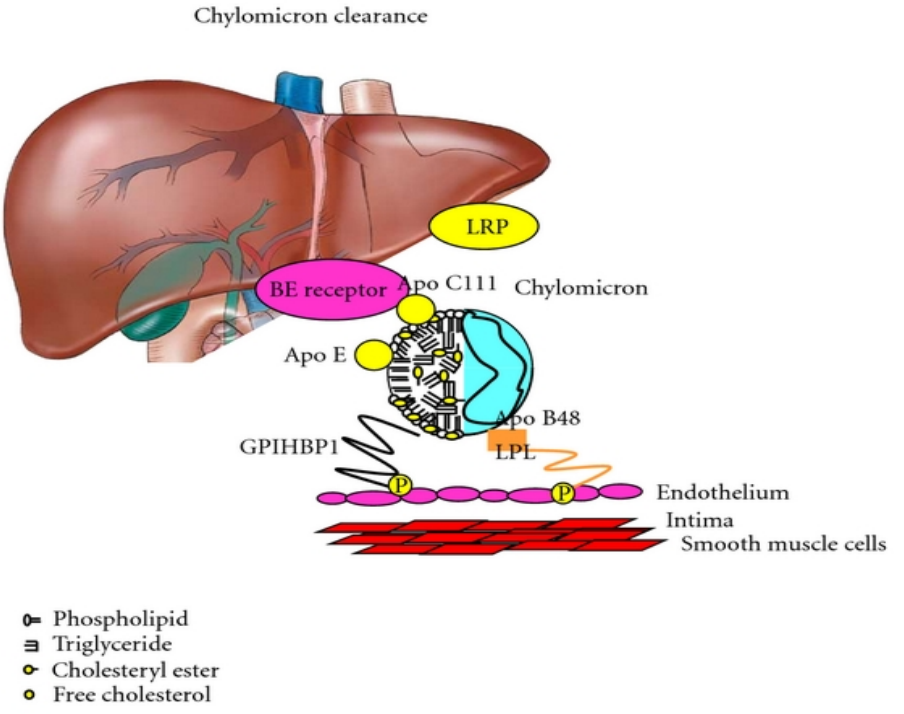


Figure 3. Chylomicron clearance by liver[1] (Tomkin and Owens, 2012)

[1]Apo C111, Apoliprotein C111; Apo B48, Apoliprotein B48; Apo E, Apoliprotein E; LPL, lipoprotein lipase; GIHBP1, glycosylphosphatidyl inositol anchored high-density lipoprotein binding protein 1; LRP, LDL receptor-related protein; BE receptor, low density lipoprotein B/E receptor.

Hepatic fatty acid uptake and channeling

The liver uptakes exogenous FAs from two sources: chylomicron remnant uptake and free fatty acid (FFA) uptake from the blood. Several studies show that uptake of exogenous FFA is the single largest source of FA in stored hepatic TG which further may be one chief cause of liver fat accumulation. Under normal conditions, the majority of plasma FFA, which is mainly resulted from adipose tissue lipolysis, is bound to albumin. Traversing FFA through plasma membrane into hepatocyte is manly regulated by a protein mediated mechanism (Doege and Stahl, 2006). For hepatic FA uptake, CD36 is verified not to be required under normal conditions, while instead, liver specific Fatp5 plays the main role (Mashek, 2013). FATP5 deletion could significantly reduce the hepatic LCFA uptake (Nassir and Ibdah, 2014). While inside the cell, FFAs are rapidly coupled to CoA by the regulation of ACSLs or by the FATPs itself (Doege and Stahl, 2006). Further, highly conserved cytosolic protein, ACBP which shows high affinity binding to long-chain acyl-CoA esters, further transports acyl-CoA to mitochondria for beta-oxidation(Rasmussen et al., 1994). In addition, ACBP also plays an important role in hepatic steatosis as its transgenic overexpression resulted in increased accumulation of lipid and increased hepatic TG (Hardwick et al., 2013).

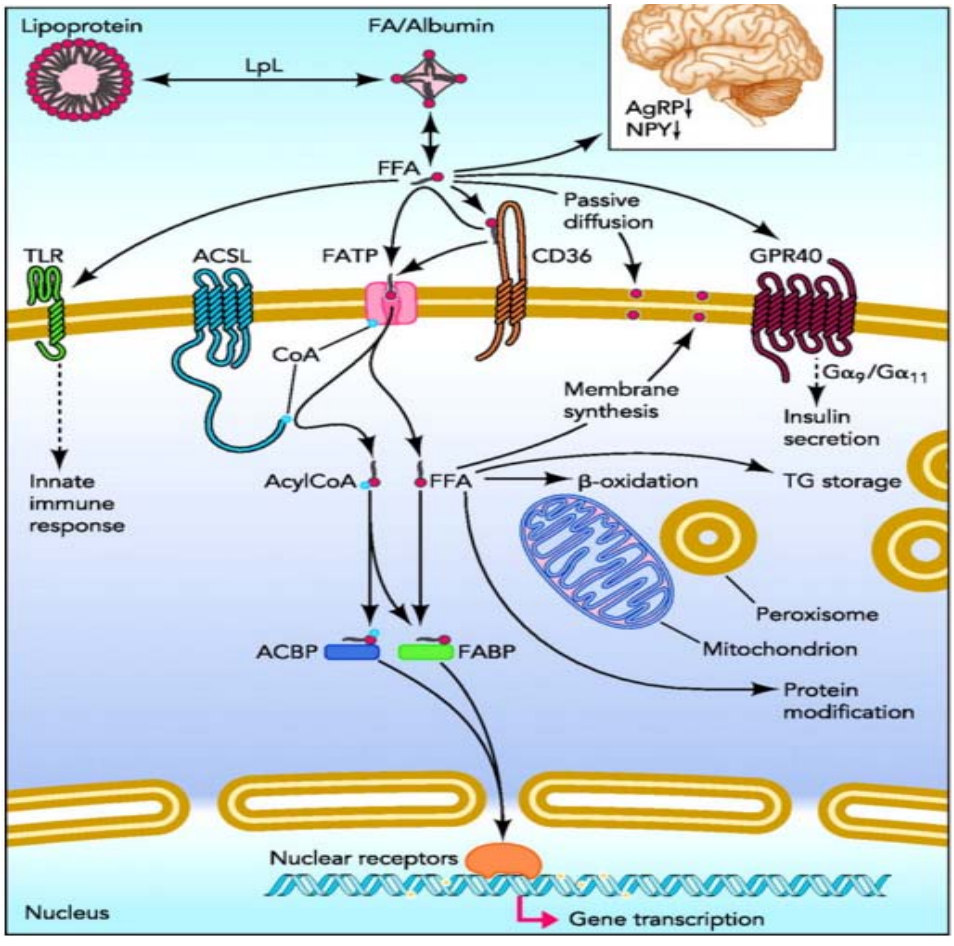


Figure 4. Free fatty acid uptake and channeling in hepatocyte[1] (Doege and Stahl, 2006).

[1]CD36, cluster of differentiation; FFA, free fatty acid; FATP, fatty acid transport protein; GPR40, G protein–coupled receptor 40; LpL, lipoprotein lipase; FABPs, Fatty acid binding proteins, ACBPs, acyl-CoA binding proteins; ACSLs, long-chain fatty acyl-CoA synthetases, TLRs, toll-like receptors; AgRP, Agouti-related protein; NPY, neuropeptide Y.

3. Hepatic lipid metabolism

Hepatic steatosis arises from imbalance in TG acquisition and removal. Therefore, except the process of plasma chylomicron remnant and FFA uptake, regulation of intracellular TG lipolysis, FA oxidation, de novo TG synthesis and regulation of TG secretion with VLDL also play important roles on determining liver fat accumulation (Cahova et al., 2012). It is reported that not only amount but also fatty acid composition may influence liver TG accumulation (Green and Hodson, 2014). For instance, mono-unsaturated fatty acid and/or n-6 unusual polyunsaturated fatty acids (PUFA) are preferentially partitioned toward oxidation pathways compared to saturated FA (Moussavi et al., 2008); PUFAs could suppress the expression of lipogenic genes and further lower liver TG accumulation (Green and Hodson, 2014).

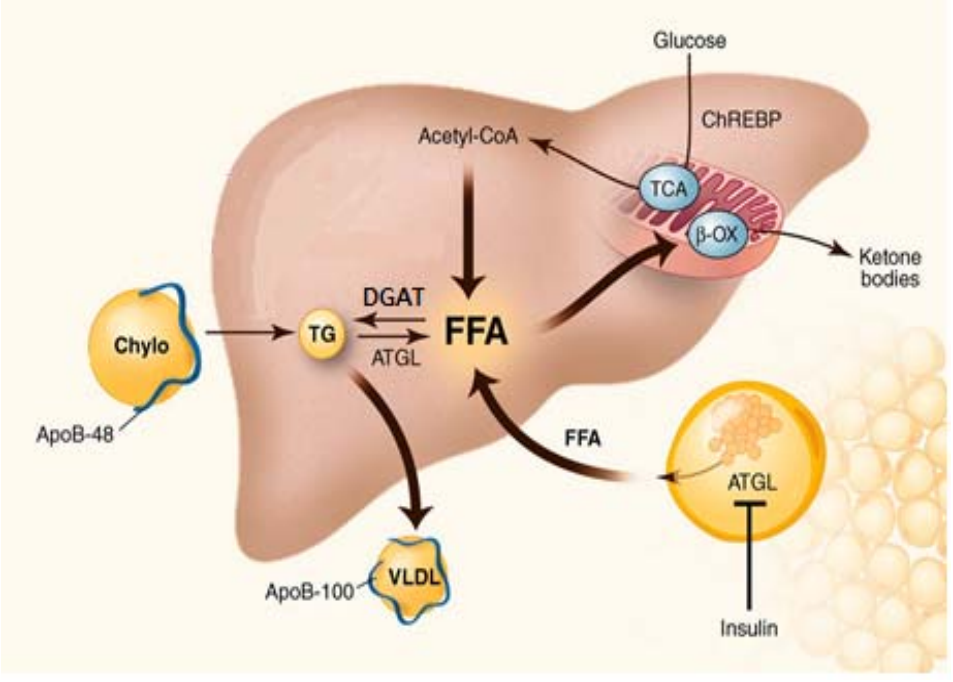


Figure 5. Metabolism of triacylglycerol in the liver[1] (Jonathan C. Cohen, 2011).

[1]Chylo, chylomicron; ATGL, adipocyte triacylglycerol hydrolase; DGAT, diglyceride acyltransferase; FFA, free fatty acid; β-OX, beta-oxidation; ChREBP, carbohydrate responsive element-binding protein; TCA, tricarboxylic acid; VLDL, very-low-density lipoproteins; ApoB-100, apolipoprotein B-100.

TG lipolysis and FA beta-oxidation

Adipocyte TG hydrolase (ATGL) is a critical regulator of TG hydrolysis in liver. Many studies indicate that besides the function of TG breakdown, ATGL also selectively channels hydrolyzes FAs to beta-oxidation and activates peroxisome proliferater-activated receptors alpha (PPARa), which is a critical transcriptional regulator of genes encoding fatty acid oxidation enzymes in liver, without affecting hepatic VLDL secretion (Ong et al., 2011; Turpin et al., 2011). Therefore, any alterations in hepatic ATGL expression or activity could result in a metabolic diseases including non-alcoholic fatty liver disease.

Hepatic fatty acid oxidation is primarily generated within mitochondria matrix and to a much lesser extent by peroxisomes and microsomes. The transportation of FA into the mitochondrial matrix for beta-oxidation is an enzyme-dependent process. Carnitine palmitoyltransferase (CPT1), which locates on the outer mitochondrial membrane, is the main regulator in this FA transportation, and is also the rate-limiting enzyme for FA oxidation (Ryu and Cha, 2003). Mitochondrial beta-oxidation is a process where FA acyl-CoA loses two carbon units at each cycle, released as acyl-CoA. This process undergoes dehydrogenation, hydration, and cleavage reactions which need a membrane-bound and soluble enzymes (Fabbrini et al., 2010). Acyl-CoA Dehydrogenase, Long Chain (ACADL), Enoyl-CoA, Hydratase/3-Hydroxyacyl CoA Dehydrogenase (EHHADH) and Acetyl-CoA Acyltransferase 1 (ACAA1) are three marker enzymes in mitochondrial beta-oxiation

(Guo et al., 2007; van der Leij et al., 2007). ACADL functions in the first step to shorten long-chain FA acyl-CoA. EHHADH catalyzes the second and third steps of mitochondrial beta-oxidation, hydration and dehydrogenation of enoyl-CoAesters to ketoacyl-CoA. While ACAA1 catalyzes the last step in the cycle by cleaving 3-oxoacyl-CoA to acetyl-CoA (Cahova et al., 2012).

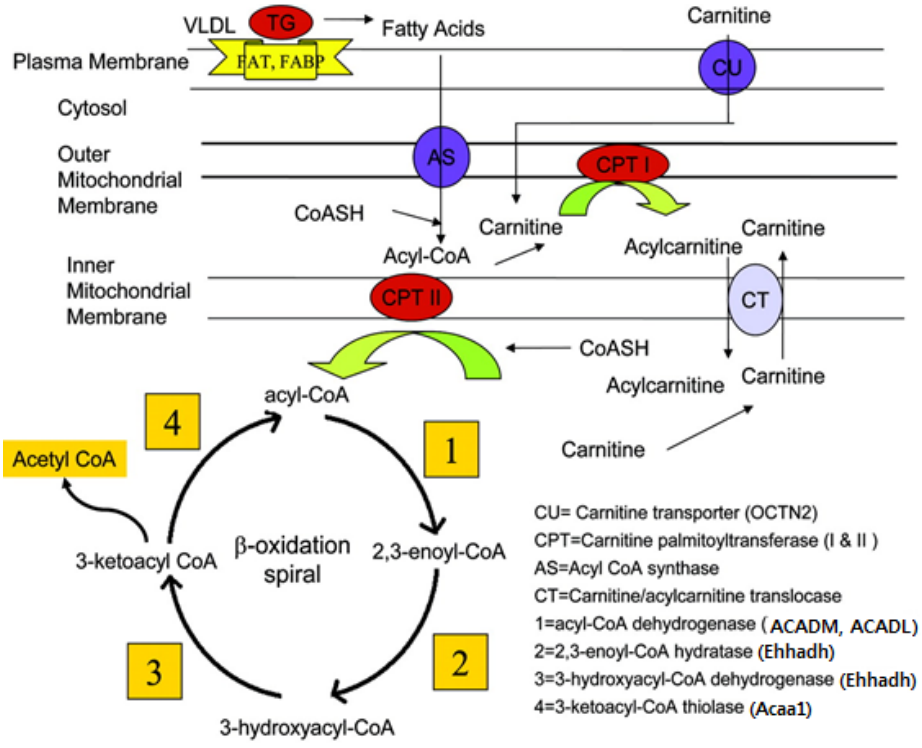


Figure 6. The mitochondrial fatty acid oxidation (FAO) pathway[1] (Shekhawat et al., 2003).

[1]FAT, fatty acid transporter; FABP, fatty acid-binding protein; TG, triglycerol; VLDL, very low-density lipoprotein; ACADM, medium-chain acyl-CoA dehydrogenase; ACADL, long-chain acyl-CoA dehydrogenase; Ehhadh, Enoyl-CoA, Hydratase/3-Hydroxyacyl CoA Dehydrogenase; Acaa1, Acetyl-CoA Acyltransferase 1.

Hepatic TG synthesis

Diacylglycerol acyltransferases (DGAT) transfers FA-acyl-CoA to DG to form TG, which is the terminal step in TG synthesis. There are mainly two DGAT enzymes exist in a wide variety of eukaryotes, DGAT1 and DGAT2, while DGAT2 is a more potent DGAT with a higher affinity for its substrates than DGAT1, and appears to be the dominant DGAT enzyme controlling TG homeostasis in vivo (Yen et al., 2008). It is verified that the overexpression of DGAT in liver led to increased hepatic TG synthesis without changing VLDL production (Millar et al., 2006).

Hepatic VLDL secretion

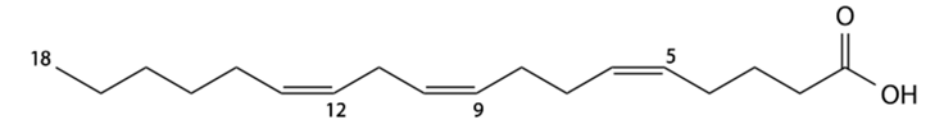
Very-low-density lipoproteins (VLDL) assembly in liver involves the fusion of TG and a newly synthesized apolipoprotein B-100 (ApoB-100) by aid of MTP. Each VLDL particle contains a single molecule of ApoB100 so apoB100 is regarded as an indispensable structural protein in VLDL assembly. The formation of VLDL particles in the liver is an important process because it converts the liposoluble TG into a hydrosoluble form that can be transported from the liver to peripheral tissues (Fabbrini et al., 2010). Besides rapid incorporation of dietary FA into VLDL formation also enables dietary FAA to be recycled for further uptake by extrahepatic tissue (Heath et al., 2003).

4. Characteristics of pine nut oil

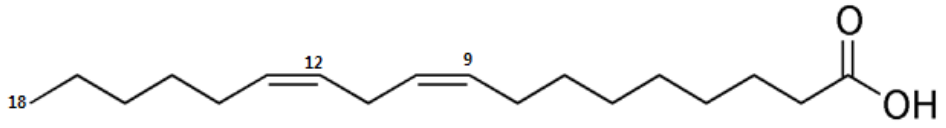
Vegetable oils from the seeds of some conifers, such as *Pinus pinaster* and *Pinus koraiensis,* are used for a long time in the feed industry or as dietary supplements (Imbsa et al., 1998; Lee et al., 2004). For instance, the oil produced from the seeds of some European and American pine varieties is mostly used for culinary, medicine and other purposes (Östlund et al., 2009; Svanberg et al., 2012).

These vegetable oils are reported to be special because to a certain degree, it contains some PUFA which are typified by poly-methylene interrupted double bonds. Many studies have shown that PUFA has numerous favorable effects such as influencing many parameters of the immune functions (Erickson et al., 1983; Matsuo et al., 1996), or reducing plasma lipid levels (Lee et al., 2004).

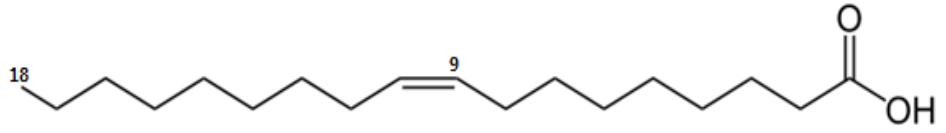
However, among all kinds of PUFA contained nuts, only a few of them can exert biologically activity. To this regard, the oil from *Pinus koraiensis,* also known as pine nut oil or *pine seed oil*, has appealed researchers attention for a long time; as it contain with the highest content of pinolenic acid ($18:3^{\Delta 5,9,12}$) (Imbsa et al., 1998), which has an unusual UPIFA with a cis 5 ethylenic bone. Besides highly content of pinolenic acid (14.9%) , which accounts for most of the Δ-UPIFA (17.7%) content, the other main FA in pine nut oil are linoleic acid ($18:2^{\Delta 9,12}$, 48.4%), oleic acid ($18:1^{\Delta 9}$, 25.5%) (Lee et al., 2004).



Pinolenic acid (all-cis-18:3$^{\Delta 5,9,12}$)



Linoleic acid (all-cis-18:2$^{\Delta 9,12}$)



Oleic acid (cis-18:1$^{\Delta 9}$)

Figure 7. Structures of Pinolenic acid, Linoleic acid and Oleic acid.

The health beneficial properties of PNO have been studied for years. It has reported that Korean pine nut may function to suppress appetite by increasing satiety hormones level such as CCK-8 and thus reducing food intake (Pasman et al., 2008). Dietary PNO supplementation increased Ig production from spleen lymphocytes which indicated PNO's influence on immune function (Matsuo et al., 1996). Effects of PNO on lipid profile have also appealed considerable interests for a long time. Ferramosca et al. (Ferramosca et al., 2008) showed that mice fed with PNO-containing diet resulted in lower body weight gain, liver weights, and total serum cholesterol and TAG levels in comparison with mice fed with maize oil-contained diet. Consumption of a diet containing PNO in mice also resulted in lower serum TAG and VLDL-TAG levels compared with mice fed diets containing other oils such as sunflower or linseed oils (Asset et al., 1999).

Recent studies started focusing their attention on the role of PNO in the modulation of obesity. PNO replacement in the diet was shown to be effective in lowering body weight gain and liver weight in HFD induced obese mice (Park et al., 2013). In a study by Le et al. (Le et al., 2012), enhanced mitochondrial FA oxidative metabolism in skeletal muscle and brown adipose tissue was observed in obese mice with PNO consumption evidenced by higher expression of genes related to FA oxidation such as *Ppara*, *Cpt1* and *Acadl*.

III. Materials and Methods

1. Animals and diets

Five-week-old male C57BL/6N mice were purchased from Central laboratory animal Inc. (Seoul, Korea) and fed a SC-control diet for 3 days before divided into four dietary groups. Control diets contain 10% kcal fat from PNO (PC, n=11) or SBO (SC, n=10) and high-fat diets contain 15% kcal fat from lard and 30% kcal fat from PNO or SBO (PHF or SHF, both n=12). The diets were made by Dyets, Inc. (Bethlehem, Pennsylvania, USA). **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. (Hwaseong-City, GyeongGi-do, Korea). Mice were housed individually in a pathogen-free facility under a controlled environment that provided constant temperature (23 ± 3°C) and humidity (55 ± 10%) and a light/dark cycle (12 h/12 h). Body weight was measured every week. At the end of 12 weeks, the animals were euthanized with CO_2 asphyxiation after 12 hour fasting. Blood was collected by heart puncture and serum was isolated from blood by centrifugation at 3000 rpm for 20 minutes at 4°C. Small intestine and livers were dissected out, and with the remove of surrounding fat, small intestine was divided into duodenum, ileum and jejunum. White adipose tissue, which located behind the kidney and along the back of the abdomen were also dissected. All tissues and serum were immediately snap-frozen in liquid nitrogen and stored at -80°C for later biochemical and molecular analysis. All the exper-

iment procedures involving mice were conducted in accordance with the guideline approved by Institutional Animal Care and Use Committee of Seoul National University (approval No. SNU-101029-1)

Table 1. Composition of the experiment diets[1]

	Control diet (10% Oil)	High-fat diet (30% Oil+15% Lard)
casein (g)	200	200
L-cystine (g)	3	3
Sucrose (g)	350	172.8
Cornstarch (g)	315	72.8
Dyetrose (g)	35	100
PNO[2] or SBO (g)	45	135
Lard (g)	0	37.5
t-Butylhydroquinone (g)	0.009	0.027
Cellulose (g)	50	50
Mineral mix (g)[3]	35	35
Vitamin mix (g)[4]	10	10
Choline bitartrate (g)	2	2
Total (g)	1045.0	848.1
kcal/g diet	3.7	4.6

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

[2] PNO was a gift from the Dubio Co., Ltd. (Hwaseong-City, GyeongGi-do, Korea)

[3]Thirty-five grams of mineral mix (Dyets, #210099) provides 5.1 g calcium, 4 g phosphorus, 3.6 g potassium, 1 g sodium, 1.6 g chloride, 0.5 g magnesium, 0.3 g sulfur, 59 mg manganese, 46 mg iron, 25 mg zinc, 5 mg copper, 0.2 mg selenium, 0.2 mg iodine and 4.2 g sucrose.

[4]Ten grams of vitamin mix (Dyets, #300050) 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, 0.8 mg menadione, 0.2 mg biotin, 10 μg vitamin B12 and 9.8 g sucrose.

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

	Control diet		High fat diet	
	Soybean oil (SC)	Pine nut oil (PC)	Soybean oil (SHF)	Pine nut oil (PHF)
Myristic acid (C14:0)	ND[2]	ND	0.4	0.4
Palmitic acid (C16:0)	11.9	7.0	14.0	10.5
Stearic acid (C18:0)	4.8	3.6	6.9	6.2
Total saturated fatty acid	16.7	10.6	21.3	17.1
Palmitoleic acid (C16:1, Δ9)	ND	ND	0.6	0.7
Oleic acid (C18:1, Δ9)	21.1	27.4	27.7	31.7
Total monounsaturated fatty acid	21.1	27.4	28.3	32.4
Linoleic acid (C18:2, Δ9, 12)	54.9	47.2	44.9	39.7
α-linolenic acid (C18:3, Δ9, 12, 15)	7.4	0.8	5.5	1.0
Pinolenic acid (C18:3, Δ5, 9, 12)	ND	14.0	ND	9.7
Total polyunsaturated fatty acid	62.3	62.0	50.4	50.4

[1]The extraction methods will be shown later in Materials and Methods 2. Fatty acid composition of the experimental diets.

[2]ND, not detected

2. Fatty acid composition of the experiment diets

Total lipids were extracted from the experimental diet using a Folch extraction protocol (Folch et al., 1957). 50 mg diet samples were dissolved with 1mL chloroform and 0.5 mL methanol. After dispersion, the whole mixture was agitated on a tube rocker for 16 hours at room temperature. Then the homogenate was centrifuged at 2000 rpm for 10 minutes and 1 mL supernatant was removed to a new tube. Before another centrifugation at 2000 rpm for 10 minutes, added to the supernatant was agitated with 0.2 mL of 0.9% NaCl for 30 minutes. Then the lower layer containing lipids was removed to a new tube and was evaporated under vacuum under a nitrogen stream.

By methylating the lipid sample extracted through the above process, fatty acid methyl ester was generated. Then the rest lipids were reconstituted in 0.4 mL of 0.5 M methanolic NaOH and incubated at 100°C for 5 minutes and 0.4 mL of fresh 14% BF_3 in methanol was added. Another incubating process at 100°C for 5 minutes was performed. After that, 8.5 mL H_2O and 0.5 mL hexane are added to the sample and the mixture was incubated for 10 minutes at room temperature before performing centrifugation for 5 minutes under 2000 rpm. After centrifugation, the supernatant was removed and kept for gas chromatography (GC) analysis.

10% of samples (1 uL sample solution with split ratio 1:10) were measured with GC machine (Agilent 7890A, Agilent Technologies, Santa Clara, California, USA) using DB-Carbowax column (0.32 mm × 25 m, 0.2 μm, Agilent Technologies, San-

ta Clara, California, USA) and flame ionization detector. Helium was used as carrier gas with 1.5 mL/min constant flow compensation, injection temperature was raised from 50°C to 200°C at 15°C/min and maintained for 20 minutes at 200°C. By comparing peaks with the compound retention time, we could get total FA composition in diet sample. And the proportions of each fatty acid contained in the sample were determined by calculating the ratio of a single peak area (representing one specific fatty acid) to the total peak area.

3. Serum lipid analysis

Serum TG level was measured enzymatically using Cleantech TG-S kit (Asan pharm Co., Ltd, Seoul, Korea) using a series of coupled reactions where TG were hydrolyzed to produce fatty acids and glycerol. Glycerol then undergoes phosphorylation and oxidation, and H_2O_2, one of the reaction products can be measured quantitatively in a peroxidase catalyzed reaction that produces a color. The absorbance of the color can be later used to calculate serum TG concentration. In this study, 300 uL of enzyme solution and 2 uL of serum sample or standard sample (300 mg/dL of glycerol) were added to 96-well plates with 10 minutes incubation at 37°C. The absorbance was measured using a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, California, USA) at 550 nm. Serum TG concentration was calculated based on the standard curve derived from the absorbance of the standard sample.

Serum NEFA concentration was measured by SICDIA NEFAZYME Kit (Shin Yang Chemical, Busan, Korea). This measurement is similar with serum TG content measurement which is also based on an enzymatic assay. Acyl coenzyme A is produced from free fatty acid, and it then goes through a re-oxidizing enzymatic process, producing H_2O_2 which can induce a color-change-reaction. In this study, 4uL serum sample or standard solution and 200 uL of NEFA reagent 1 were added to each well of the 96-well microtiter plate and the mixture was incubated at 38°C for 5 minutes. Later another NEFA reagent 2 was added to each well by 100 uL and

the absorbance was measured under 546 nm wavelengths using a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, California, USA). Serum NEFA concentration was calculated based on the standard curve derived from the absorbance of the standard sample.

4. Measurement of hepatic lipid contents

Folch method was used to extract total lipids from liver (Folch et al., 1957). 25 mg liver tissue was homogenized in 2 mL tube with 60 uL PBS solution. Then 800 uL chloroform and 400 uL methanol were added to the homogenate and the mixture was incubated for 16 hours at room temperature. Then the homogenate was centrifuged at 2000 rpm for 10 minutes at room temperature and the supernatant afterwards was removed to a new tube. After that, the cap of the tube was left open in a fume hood for 3 hours and the remaining solid was well dissolved in 0.1 mL isopropanol. Measurement of liver TG level was carried out in the same way as mentioned in method 3.

5. RNA extraction and cDNA synthesis

Total RNA was extracted from jejunum and liver using TRIzol reagent (Invitrogen, Carlsbad, California, USA) following the manufacturer's instruction. Frozen intestine and liver samples (~50 mg) were homogenized in 1ml of Trizol reagent by a power homogenizer (IKA T10 Basix Ultra-turrax, IKA, Königswinter, Germany). The homogenates were incubated for 5 minutes at room temperature before supplementing it with 0.2 mL of chloroform. After being shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes, subsequently the homogenized samples were centrifuged at 12000 g for 15 minutes at 4°C. Following centrifugation, since RNA remains exclusively in the colorless upper aqueous phase whereas DNA and proteins are in the interphase and organic phase, the aqueous phase was then mixed with 0.5 mL of isopropyl alcohol in a new tube, incubating at room temperature for another 10 minutes. The new mixture was centrifuged at 12000 g for 10 minutes at 4°C, then the supernatant were discarded, and the RNA pellets was washed with 1 mL of 75% ethanol. After vortexing, the samples were centrifuged at 7500 g at 4°C for 2 minutes, and again the supernatant were removed to let RNA pellets briefly dry. In the end, 20 uL of diethylpyrocarbonate (DEPC) water was added to redissolve RNA pellet. Isolated RNA quantity and quality were determined via spectrophotometry using a spectrophotometer (Opitzen 2120UV, Mecasys Co., Ltd, Daejeon, Korea) and by agarose gel electrophoresis using Gel-Dox XR system (Bio-Rad Laboratories, Inc., Berkeley, California, USA), respectively.

The extracted RNA with the final concentration of 0.5 ug/uL was used for cDNA synthesis with PrimeScript II 1st strand cDNA synthesis kit (Takara Bio Inc., Otsu, Shiga, Japan). A RNA primer mixture with total RNA 2 μg, oligo dT primer (50 mM) 1 μL, dNTP mixture (10 mM) 1 μL, RNase-free dH_2O 3 μL was incubated at 65°C for 5 minutes. With another 5 minutes' ice incubation, PrimeScript II buffer (5×) 4 μL, RNase inhibitor (40 U/μL) 0.5 μL, PrimeScript II RTase (200 U/μL) 1 μL and RNase-free dH_2O 4.5 μL were added to RNA primer mixture, making the reaction mixture with a total volume of 20 uL. Thermal Cycler 2720 (Applied Biosystems, Foster, California, USA) was then used to perform cDNA synthesis with the following conditions: 42°C for 50 minutes, 95°C for 5 minutes and 4°C for 30 minutes. The synthesized cDNA was preserved at -20°C for future use.

6. Real-time polymerase chain reaction (PCR) analysis

Real-time PCR was performed in StepOne Real-time PCR System (Applied Biosystems, Foster, California, USA) with a SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan). PCR measurements were performed in a total volume of 20 uL, containing 0.4 uL of 10 uM each primer, 0.4 uL of ROX reference dye, 10 uL of SYBR Premix Ex Taq, 1uL of 2 ng/uL cDNA and 7.8 uL of autoclaved distilled water. The following PCR program was carried out: 95°C for 30 seconds to initial denaturation, 40 cycles of 95°C for 10 seconds to denaturation and 60°C for 30 seconds to annealing, extension, reading fluorescence. Calculations were performed by a comparative method ($2^{-\Delta\Delta CT}$) using housekeeping gene *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) as an endogenous control. StepOne software (version 2.1, Applied Biosystems, Foster, California, USA) was used to measure amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software also determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample. Specific primer sequences used in this study are shown in **Table 3** and **Table 4**.

Table 3. The primer sequences for intestinal genes used in real-time PCR

Gene	Function	Forward primer (5'- 3')	Reverse primer (5'- 3')
Fatp4	fatty acid uptake	GTGGTGCCCGATGTGTAGAT	CTCAGCCTCTTCTGCTCTGG
Cd36	fatty acid uptake	CCAAGCTATTGCGACATGATT	TCTCAATGTCCGAGACTTTTCA
Acsl5	fatty acid metabolism	GGCCAAACAGAATGCACAG	GGAGTCCCAACATGACCTG
Acbp	fatty acid transporter	AGGTTAACGCTGGCCCTAAT	TGCCATGAAGACCTATGTGG
Mtp	chylomicron assembly	TCTGGCTGAGGTGGGAATAC	CACTCAGGCAATTCGAGACA
ApoB48	chylomicron assembly	TGAATGCACGGGCAATGA	GGCATTACTTGTTCCATGGTTCT
ApoA4	chylomicron secretion	TTCCTGAAGGCTGCGGTGCTG	CTGCTGAGTGACATCCGTCTTCTG

Fatp4: Fatty acid transporter 4; *Cd36*: Cluster of differentiation 36; *Acsl5*: acyl-CoA synthetase long-chain family member 5; *Acbp*: Acyl-CoA-binding protein; *Mtp*: microsomal triglyceride transfer protein; *ApoB48*: apolipoprotein B-48; *ApoA4*: apolipo-protein A-IV.

Table 4. The primer sequences for hepatic genes used in real-time PCR

Gene	Function	Forward primer (5'- 3')	Reverse primer (5'- 3')
Lrp1	chylomicron remnant receptor	GACCAGGTGTTGGACACAGATG	AGTCGTTGTCTCCGTCACACTTC
Fatp5	fatty acid uptake	GAATCGGGAGGCAGAGAACT	AGCGGGTCATACAAGTGAGC
Acsl1	fatty acid channeling	CACTTCTTGCCTCGTTCCAC	GTCGTCCCGCTCTATGACAC
Acbp	fatty acid channeling	AGGTTAACGCTGGCCCTAAT	TGCCATGAAGACCTATGTGG
Atgl	TG lipolysis	TGTGGCCTCATTCCTCCTAC	TCGTGGATGTTGGTGGAGCT
Cpt1a	mitochondrial β-oxidation	GATGTTCTTCGTCTGGCTTGA	CTTATCGTGGTGGTGGGTGT
Acadl	mitochondrial β-oxidation	TCGCAATATAGGGCATGACA	ACTTGGGAAGAGCAAGCGTA
Ehhadh	mitochondrial β-oxidation	ATGGCTGAGTATCTGAGGCTG	ACCGTATGGTCCAAACTAGCTT
Acaa1	mitochondrial β-oxidation	CTGTAGCGTCCCTCTCTGGA	AGCAAGGCAGGTTGTCACG
Dgat2	TG synthesis	CTTCCTGGTGCTAGGAGTGG	GCCAGCCAGGTGAAGTAGAG
ApoB100	VLDL assembly	TGAATGCACGGGCAATGA	GGCATTACTTGTTCCATGGTTCT

Lrp1: low density lipoprotein receptor-related protein 1; *Fatp5*: Fatty acid transporter 5; *Acsl1*: acyl-CoA synthetase long-chain family member 1; *Acbp:* Acyl-CoA-binding protein; *Atgl*: Adipose triglyceride lipase; *Cpt1a*: carnitine palmitoyltransferase 1a; *Acadl*: Long Chain Acyl-CoA Dehydrogenase; *Ehhadh*: enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase; *Acaa1*: acetyl-Coenzyme A acyltransferase 1a; *Dgat2*: diacylglycerol O-acyltransferase 2; *ApoB100*: apolipoprotein B-100

7. Statistical analysis

The overall effects of fat amount and oil type, and the interaction between the two were examined by Two-way ANOVA. The individual group comparison were determined by Fisher's LSD multiple comparison test. All statistical analysis was performed within SPSS software version 23 statistical package for Windows (SPSS Inc., Chicago, Illinois, USA). The results were expressed as means ± SEM and significance was declared at $P<0.05$.

IV. Results

1. Body weight, weight gain, white adipose tissue weight and liver weight

As shown in **Table 5**, body weight ($P<0.05$), weight gain ($P<0.05$) and amount of white adipose tissue (WAT) ($P<0.05$) were significantly higher in HFD fed mice in comparison to control diet fed mice. The replacement of SBO with PNO showed significant effect on weight gain and WAT weight. PNO-fed groups had significantly lower weight gain ($P<0.05$) and amount of WAT ($P<0.05$) than SBO-fed groups. PHF group had significantly lower body weight gain (15.9% lower, $P<0.05$) and WAT amount (19.9% lower, $P<0.05$) than SHF group. Weekly body weight change curve is shown in **Fig 8**. From where we could see weight gain continued thereafter to be lower in PNO fed mice. Lower WAT amount in PC group compared with SC group (29.7% less, $P<0.05$) was also observed. PNO-fed mice had significantly lower liver weight in comparison to SBO-fed mice ($P<0.05$). PHF group had significantly lower liver weight than SHF group (12%, $P<0.05$). No differences were observed in liver-to-body weight percentage between SHF and PHF groups.

Table 5. Body weight, weight gain, white adipose tissue weight and liver weight[1]

	Control		High-fat		Fat amount (*P*-value)	Oil type (*P*-value)	Interac-tion (*P*-value)
	SC	PC	SHF	PHF			
Body weight at 0 wk (g)	17.28±0.85	16.74±0.45	16.84±0.37	16.75±0.44	0.62	0.49	0.62
Body weight at 12 wk (g)	32.76±0.96^{ab}	30.51±0.64^{a}	38.02±1.15^{c}	34.58±1.16^{b}	0.00	0.01	0.57
Body weight gain (g)	15.48±0.85^{ab}	13.76±0.59^{a}	21.19±0.87^{c}	17.83±1.14^{b}	0.00	0.01	0.37
White adipose tissue (g)[2]	3.10±0.22^{b}	2.18±0.18^{a}	5.28±0.32^{d}	4.23±0.33^{c}	0.00	0.00	0.82
Liver weight(g)	1.18±0.05^{b}	1.09±0.03^{ab}	1.13±0.04^{b}	0.99±0.03^{a}	0.04	0.00	0.56
Liver-to-body weight percentage(%)[3]	38.13±0.61^{b}	38.23±0.63^{b}	31.08±0.55^{a}	29.95±0.53^{a}	0.00	0.38	0.29

Data are presented as means ± SEM, n = 10-12 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]White adipose tissue contains epididymal fat, abdominal fat, perinephrium fat and subcutaneous fat.

[3]Liver-to-body weight percentage (%) = 100% * liver weight (g)/ body weight (g)

SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 30% soybean oil + 15% lard; PHF, 30% pine nut oil + 15% lard.

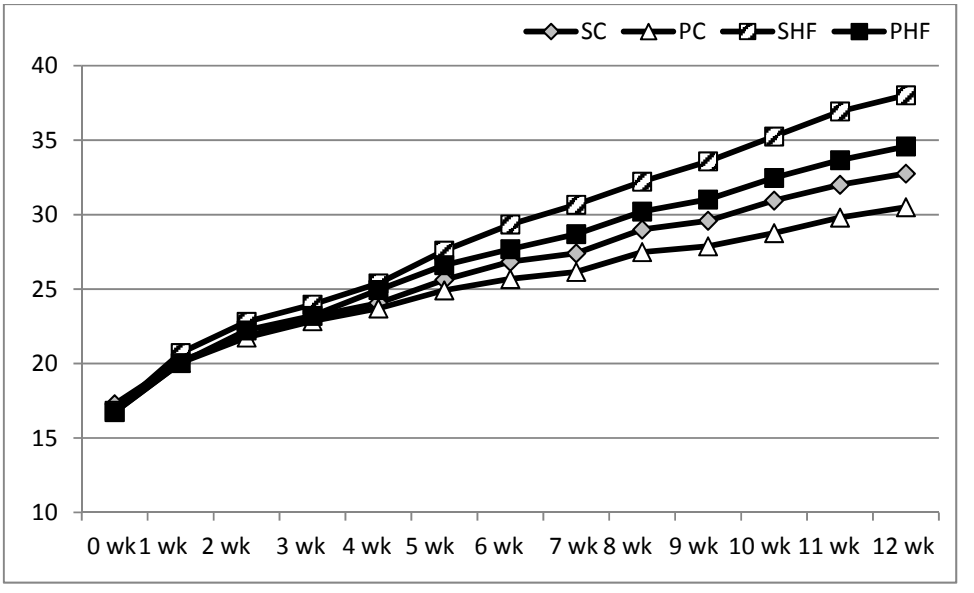


Figure 8. Body weight curves of mice fed with control or HFD containing SBO or PNO.

SC (n=10), 10% soybean oil; PC (n=11), 10% pine nut oil; SHF (n=12), 30% soybean oil + 15% lard; PHF (n=12), 30% pine nut oil + 15% lard.

2. Serum and liver lipid concentrations

Overall, serum NEFA concentration was significantly higher in PNO-fed mice compared with SBO-fed mice ($P<0.05$). Whereas serum TG concentration was not affected by either fat amount or oil type. HFD groups tended to have higher liver TG concentration ($P=0.09$). No significant difference was detected in liver TG level regardless of oil type (**Table 6**).

Table 6. Serum and liver lipid concentrations[1]

	Control diet		High-fat diet		Fat amount	Oil type	Interaction
	SC (n=10)	PC (n=11)	SHF (n=12)	PHF (n=12)	(*P*-value)	(*P*-value)	(*P*-value)
Serum NEFA[2] (mM)	1.09 ± 0.08[a]	1.35 ± 0.08[ab]	1.14 ± 0.05[ab]	1.58 ± 0.29[b]	0.41	0.04	0.60
Serum TG (mg/dL)	116.0 ± 8.4	133.5 ± 10.4	117.5 ± 5.3	123.0 ± 19.0	0.72	0.36	0.63
Liver TG (mg/g tissue)	29.3 ± 2.3	31.4 ± 2.8	36.0 ± 3.1	34.4 ± 2.8	0.09	0.92	0.51

Data are presented as means ± SEM, n = 10-12 for each group.

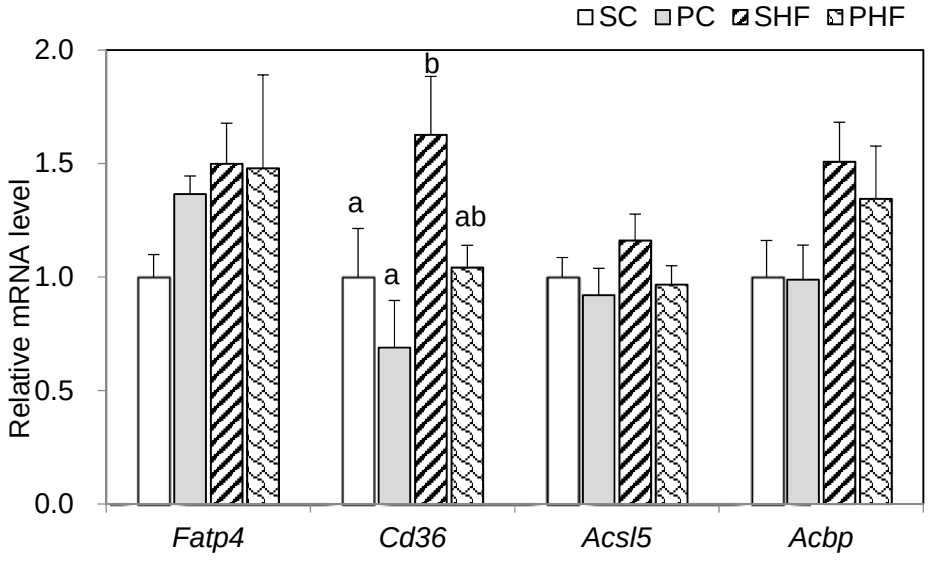
[1]Two-way ANOVA was used to determine the significant effect of fat amount and oil type. Different superscripts indicate significant differences at $P < 0.05$ by Fisher's LSD multiple comparison test.

[2]NEFA=non-esterified fatty acid

SC, 10% soybean oil; PC, 10% pine nut oil; SHF, 30% soybean oil + 15% lard; PHF, 30% pine nut oil + 15% lard

3. Expression of genes involved in intestinal fatty acid uptake and channeling

To examine whether PNO replacement in diet affects intestinal lipid absorption, the expression of genes related to fatty acid absorption, *Cd36* and *Fatp4*, and the genes involved in fatty acid channeling within enterocyte, *Acsl5* and *Acbp*, were measured (**Fig. 9**). Overall, the mRNA levels of *Cd36* were significantly higher in HFD-fed mice ($P<0.05$) but significantly lower in PNO-fed mice ($P<0.05$). PHF group had a tendency of lower *Cd36* mRNA level than SHF group (0.64-fold, $P=0.06$). Feeding HFD led to a significant higher *Acbp* gene expression ($P<0.05$), but no statistical difference was found regarding different oil type. The mRNA levels of *Fatp4* and *Acsl5* were not influenced by amount of fat and type of oil.



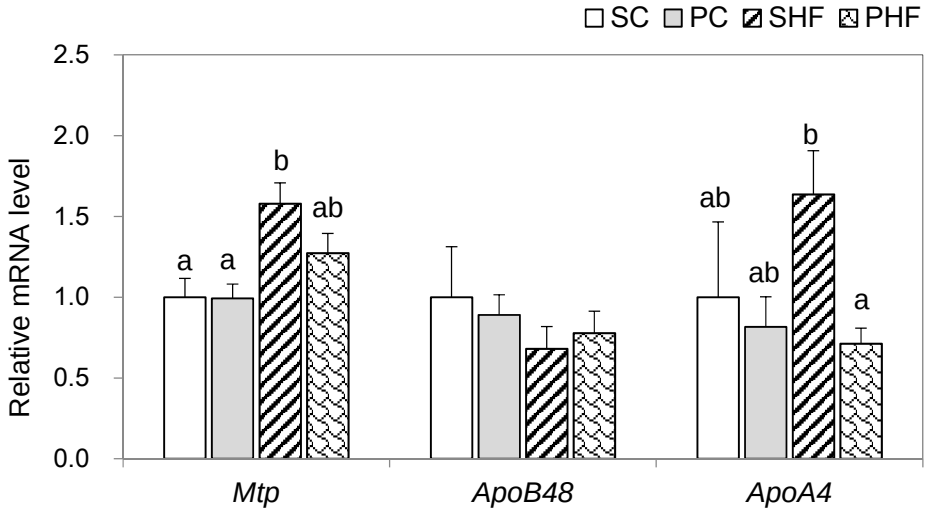
Fat amount (*P*-value)	0.20	0.04	0.32	0.03
Oil type (*P*-value)	0.47	0.03	0.19	0.64
Interaction (*P*-value)	0.42	0.51	0.58	0.68

Figure 9. The mRNA levels of genes related to intestinal fatty acid uptake and channeling.

Data are presented as means ± SEM, n=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. SC: 10% soybean oil; PC: 10% pine nut oil; SHF: 30% soybean oil+15% lard; PHF: 30% pine nut oil+15% lard. *Fatp4*: Fatty acid transporter 4; *Cd36*: Cluster of differentiation 36; *Acsl5*: acyl-CoA synthetase long-chain family member 5; *Acbp*: Acyl-CoA-binding protein.

4. Expression of genes involved in intestinal chylomicron secretion

To determine whether PNO's effect on weight gain reduction was associated with changes in chylomicron secretion, we measured the expression of genes involved in intestinal chylomicron assembly and secretion. The mRNA level of *Mtp*, an enzyme involved in chylomicron synthesis and secretion, was significantly up-regulated in HFD-fed mice ($P<0.05$). PHF group tended to have a lower *Mtp* mRNA level than SHF group (0.81-fold, $P=0.08$). The gene expression of chylomicron structural components *ApoB48* and *ApoA4*, which also facilitate intestinal lipoprotein production, were also measured. Although ANOVA result didn't show the significant difference in the mRNA levels of *ApoA4*, overall tendency of lower expression in PNO-fed mice compared with SBO fed mice ($P=0.07$) was observed. When individual group comparisons were done, PHF group had a significantly lower mRNA levels of *ApoA4* than SHF group ($P<0.05$). Neither fat amount nor oil type influenced mRNA levels of *ApoB48* (**Fig. 10**).



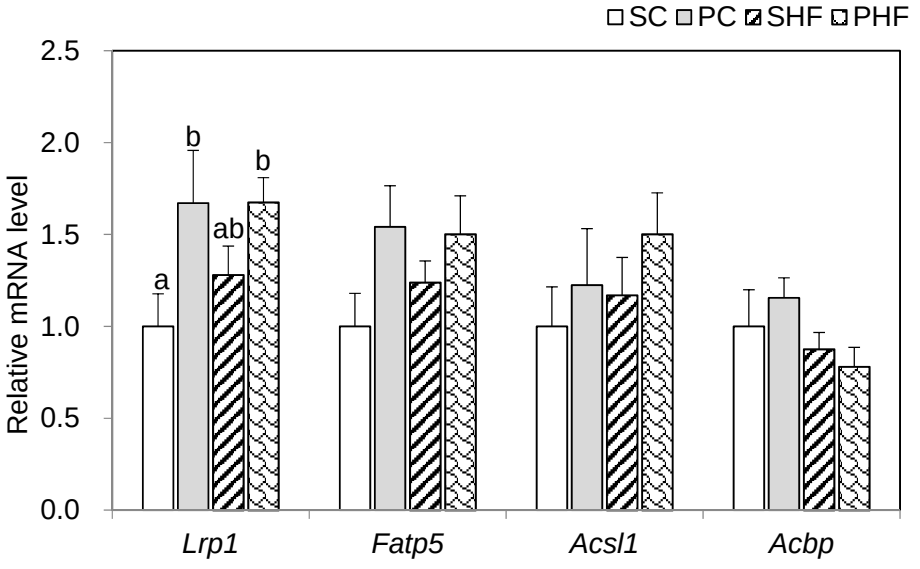
Fat amount (*P*-value)	0.00	0.28	0.37
Oil type (*P*-value)	0.19	0.97	0.07
Interaction (*P*-value)	0.21	0.60	0.22

Figure 10. The mRNA levels of genes related to intestinal chylomicron assembly and secretion.

Data are presented as means ± SEM, n=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. SC: 10% soybean oil; PC: 10% pine nut oil; SHF: 30% soybean oil+15% lard; PHF: 30% pine nut oil+15% lard. *Mtp*: microsomal triglyceride transfer protein; *ApoB48*: apolipoprotein B-48; *ApoA4*: apolipoprotein A-IV.

5. Expression of genes involved in hepatic lipid uptake and channeling

To examine whether PNO influenced hepatic lipid uptake, the mRNA levels of chylomicron remnant receptor *Lrp1* and FA uptake protein *Fatp5* were measured. PNO consumption led to significantly higher *Lrp1* gene expression than SBO-fed groups ($P<0.05$). Mice in PC group had significantly higher *Lrp1* mRNA level compared to mice in SC group (1.7-fold, $P<0.05$). The mRNA levels of *Fatp5* were significantly higher in PNO-fed mice than SBO-fed mice ($P<0.05$). This may indicate that PNO could accelerate serum chylomicron and FA clearance. However, the mRNA levels of *Acsl1* and *Acbp,* which are involved in hepatic FA channeling, did not show any difference in terms of either fat amount or oil type factors (**Fig. 11**).



Fat amount (*P*-value)	0.48	0.60	0.37	0.08
Oil type (*P*-value)	0.01	0.04	0.26	0.82
Interaction (*P*-value)	0.49	0.47	0.83	0.36

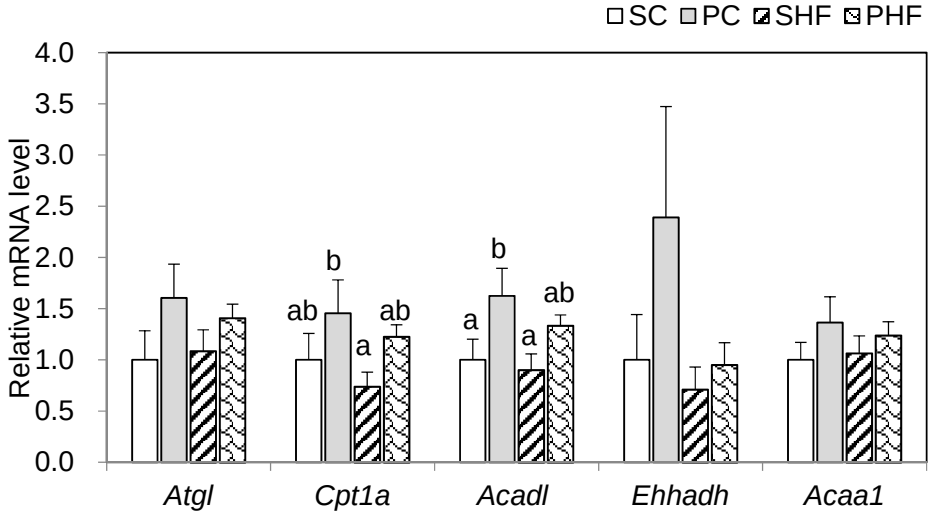
Figure 11. The mRNA levels of genes related to hepatic lipid uptake and channeling.

Data are presented as means ± SEM, n=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. SC: 10% soybean oil; PC: 10% pine nut oil; SHF: 30% soybean oil+15% lard; PHF: 30% pine nut oil+15% lard. *Lrp1*: low density lipoprotein receptor-related protein 1; *Fatp5*: Fatty acid transporter 5; *Acsl1*: acyl-CoA synthetase long-chain family member 1; *Acbp*: Acyl-CoA-binding protein.

6. Expression of gene involved in hepatic TG lipolysis and fatty acid oxidation

We also examined the expression of hepatic genes involved in TG lipolysis and FA oxidation to see whether PNO could prevent liver lipid accumulation against HFD treatment as well as increased hepatic lipid uptake. *Atgl* is known to play a key role in TG hydrolysis (Turpin et al., 2011). In current study, HFD did not make a significant difference on mRNA level of *Atgl*, whereas PNO consumption groups tended to have a higher mRNA level of *Atgl* (P=0.08).

Mitochondrial FA oxidation related genes *Cpt1a*, *Acadl*, *Ehhadh* and *Acaa1* were also measured. PNO-fed mice had higher mRNA levels of *Cpt1a* in comparison with SBO-fed mice (P=0.05). The mRNA levels of *Acadl* were also significantly higher in PNO-fed mice compared with SBO fed mice (P<0.05). Mice in PC group had significantly higher *Acadl* mRNA levels compared to mice in SC group (1.2-fold, P<0.05). The mRNA levels of *Acaa1* and *Ehhadh* were not significantly affected by the different dietary treatments (**Fig. 12**).



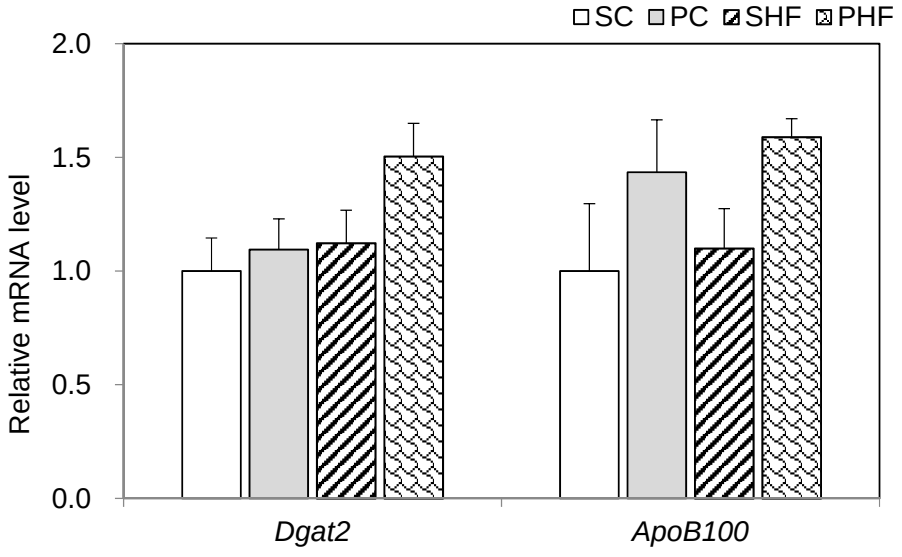
Fat amount (*P*-value)	0.83	0.29	0.32	0.17	0.87
Oil type (*P*-value)	0.08	0.05	0.01	0.19	0.16
Interaction (*P*-value)	0.58	0.95	0.61	0.35	0.62

Figure 12. The mRNA levels of genes related to hepatic TAG lipolysis and fatty acid oxidation.

Data are presented as means ± SEM, n=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. SC: 10% soybean oil; PC: 10% pine nut oil; SHF: 30% soybean oil+15% lard; PHF: 30% pine nut oil+15% lard. *Atgl*: Adipose triglyceride lipase; *Cpt1a*: carnitine palmitoyltransferase 1a; *Acadl*: Long Chain Acyl-CoA Dehydrogenase; *Ehhadh*: enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase; *Acaa1*: acetyl-Coenzyme A acyltransferase 1a.

7. Expression of genes involved in hepatic TG synthesis and VLDL assembly

TG synthesis and TG contained VLDL secretions are pivotal factors affecting lipid accumulation in liver. As shown in **Fig. 13**, compared to SBO-fed mice, PNO-fed mice had significantly higher mRNA levels of *ApoB100* which is involved in VLDL assembly and secretion ($P<0.05$). On the other hand, gene expression of *Dgat2*, an enzyme catalyzing the final step in the TG biosynthesis, was not influenced by different dietary treatments.



Fat amount (P-value)	0.08	0.56
Oil type (P-value)	0.11	0.04
Interaction (P-value)	0.33	0.90

Figure 13. The mRNA levels of genes related to hepatic TAG synthesis and VLDL assembly.

Data are presented as means ± SEM, n=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. SC: 10% soybean oil; PC: 10% pine nut oil; SHF: 30% soybean oil+15% lard; PHF: 30% pine nut oil+15% lard. *Dgat2*: diacylglycerol O-acyltransferase 2; *ApoB100*: apolipoprotein B-100.

V. Discussion

The present study showed that the replacement of SBO with PNO in control diet or in HFD resulted in lower body weight gain and less amount of WAT. PNO replacement in the diet might function to suppress excessive intestine lipid absorption by down-regulating intestinal FA uptake related genes as well as to improve hepatic lipid metabolism by up-regulating genes related to TG lipolysis, FA oxidation and VLDL secretion in both control diet and HFD fed mice.

HFD induced body weight gain is associated with excessive intestinal lipid absorption (Petit et al., 2007) and increased lipoprotein production (Huang et al., 2013). In this study, the expression of the key gene involved in intestinal FA uptake *Cd36* was significantly higher in HFD groups compared to control groups, but its expression was also significantly lower in PNO-fed groups when compared to SBO-fed groups. These results suggest that PNO replacement may prevent excessive lipid absorption from intestine.

After being taken up by enterocytes, dietary sources of lipid are used for the biosynthesis of neutral fats (Iqbal and Hussain, 2009), which are transported into the endoplasmic reticulum by *Mtp* and packaged as chylomicron with *ApoB48* and *ApoA4* (Black, 2007; Huang et al., 2013). *ApoA4* and *ApoB48* are the structural apolipoproteins of chylomicron, and the induction of *ApoA4* expression found in HFD-fed mice might also facilitate intestinal absorption as well as lipoprotein pro-

duction (Lu et al., 2006; Stan et al., 2003). In the current study, HFD groups had significantly higher mRNA level of *Mtp*, whereas PNO consumption group showed a tendency of suppression toward this overexpression. Besides, mRNA level of *ApoA4* was significantly lower in PHF group compared to SHF group. These results indicate that PNO consumption may inhibit overproduction of chylomicron by intestine in HFD fed mice.

Collectively, in intestine, PNO replacement inhibited the overexpression of genes related to intestinal FA uptake, chylomicron assembly and secretion; especially in HFD fed mice. The results suggest that PNO consumption could lower intestinal lipid absorption, chylomicron excretion and finally lead to weight loss. Since chylomicrons are responsible for the transportation of most dietary lipids from the intestinal tract into circulation (Mortimer et al., 1995), the inhibition of chylomicron secretion with PNO replacement further suggests that PNO consumption may reduce lipid flux to the circulation, liver, muscle or other lipid accumulation tissue.

Chylomicron leaves intestinal lumen, transfers dietary lipid firstly to adipose tissue or other extrahepatic tissues (Nestel et al., 1962), and delivers the remaining lipids in the form of chylomicron remnants which can be cleared from plasma by liver via *Lrp1* (Lillis et al., 2008; Masson et al., 2009). It is reported that impaired clearance of chylomicron remnants is a risk factor for the development of cardiovascular disease (Willnow, 1997) whereas this hepatic clearance can be down-

regulated by HFD (Mortimer et al., 1995). In current study, the mRNA levels of hepatic chylomicron remnant uptake receptor *Lrp1* was significantly higher in PNO-fed mice. This result indicated that PNO replacement in diet could accelerate the removal of chylomicron remnant from circulation by liver.

Dietary fatty acid can enter liver in two ways: through the uptake of chylomicron remnant derived from intestine, or through overflow of plasma free FA pool (Donnelly et al., 2005). We then measured the mRNA levels of genes related to hepatic FA uptake. It is reported that elevated plasma free FA concentration could activate the hepatic fatty acid uptake to reduce free FA levels (Adiels et al., 2008; Hardwick et al., 2009). In this study, with higher serum NEFA level observed in PNO consumption group, liver FA uptake related gene *Fatp5* mRNA level was significantly higher in PNO-fed groups. Worth to be noted, as the majority of plasma NEFA is derived from lipolysis of TG activities in WAT (Bjorndal et al., 2011; Grenier-Larouche et al., 2012). Therefore the higher serum NEFA level observed in PNO-fed group may result from the enhanced lipolysis in WAT with PNO consumption. From this point of view, additional studies may be needed to determine the influence of dietary PNO replacement on lipid metabolism and deposition in adipose tissue.

Liver lipid accumulation occurs when the amount of TG from chylomicron remnant taken from plasma and de novo lipogenesis overwhelms the amount of TG used for lipolysis and FA oxidation as well as for excretion with VLDL (Fabbrini et

al., 2010). In the current study, even though hepatic NEFA and TG uptake were increased in PNO-fed group, liver TG concentration remained similar between SBO and PNO groups. Therefore the potential mechanisms that account for liver dealing with excessive lipid influx associated with PNO consumption became our main interest. *Atgl*, in addition to its direct effect on catalyzing the initial step of TG hydrolysis, has an influence on regulation of FA oxidation without affecting VLDL secretion (Ong et al., 2011). *Cpt1a* catalyzes the rate-limiting step, shuttling FA across the mitochondrial membrane for beta-FA oxidation. *Acadl*, *Ehhadh* and *Acaa1* are three beta-oxidation marker enzymes (Guo et al., 2007; van der Leij et al., 2007). In present study, higher gene expressions of *Atgl*, *Cpt1a* and *Acadl* observed in PNO-fed groups suggest that PNO may contribute to activate TG lipolysis as well as mitochondrial FA oxidative pathway in liver.

In addition, we also measured expression of genes related to TG synthesis and secretion as VLDL. Liver-specific *Dgat2* catalyzes the terminal step in TG synthesis (Millar et al., 2006) and *ApoB100*, a structural protein on VLDL, is involved in VLDL assembly and secretion in liver (Miccoli et al., 2008). In current study, PNO consumption did not exert any influence on *Dgat2* mRNA level. However, mRNA level of *ApoB100* was significantly higher in PNO-fed groups which indicates that PNO replacement could enhance the incorporation of TG into VLDL for secretion from liver, enabling lipid to be recycled for further uptake by extrahepatic tissue.

Together, PNO replacement in diet resulted in higher plasma NEFA and chylomicron remnant clearance by liver which might relieve plasma lipid burden, but at the same time, it could reversely cause lipid accumulation in liver. However, the higher expression of genes involved in hepatic TG lipolysis, FA oxidation and VLDL production observed in PNO-fed groups indicate PNO consumption may increase hepatic lipid metabolism to accommodate the excessive hepatic lipid influx, which might further prevent a progressive liver lipid accumulation.

Even though PNO replacement resulted in less chylomicron secretion from intestine as well as elevated hepatic chylomicron remnant clearance by liver, there were still no significant differences in serum TG concentrations between two different oil dietary treatments. It is generally accepted that plasma TG has two different carriers, one is chylomicron from intestine, and another is VLDL which is mainly assembled and secreted by liver (Heath et al., 2003). Plasma TG concentrations are determined by the balance between production of chylomicron-TG and VLDL-TG (Geerling et al., 2014). Under normal conditions, chylomicron remnants could be rapidly removed from the circulation by liver (Cooper, 1997), and during postprandial period, VLDLs contributes more than 90% of TG rich lipoproteins(Heath et al., 2003). Therefore, even though PNO consumption suppressed excessive chylomicron secretion from intestine and enhanced chylomicron remnant clearance from circulation, serum TG concentration might have still remained similar between two different oil groups because of elevated VLDL secretion from liver in PNO-fed groups.

In conclusion, this study provided some evidence at transcriptional level on the disparities in various intestinal and hepatic metabolic pathways between PNO and SBO diet fed mice. PNO replacement may suppress excessive lipid absorption and chylomicron secretion into body circulation from intestine as well as enhances hepatic lipid metabolism in both control and HFD fed mice. Overall, the results may indicate PNO as potential dietary supplement for preventing metabolic dysregulations of lipids in intestine and liver seen with obesity.

VI. Summary and Conclusion

In this study, the effects of Korean pine nut oil (PNO) compared with soybean oil (SBO) on the factors involved in intestinal and hepatic lipid metabolism were investigated. After feeding mice for 12 weeks with control diets containing 10% kcal fat from SBO or PNO (SC or PC) or high-fat diets containing 45% kcal energy where 15% kcal fat from lard and 30% kcal fat from SBO or PNO (SHF or PHF), body weight, amount of white adipose tissue (WAT), serum free fatty acid and triacylglycerol (TG) levels, liver weight and TG levels were measured. Also the expression of genes involved intestinal FA uptake and channeling, intestinal chylomicron synthesis; hepatic lipid uptake and channeling, hepatic TG lipolysis and FA oxidation, as well as VLDL assembly were measured by real-time PCR. The results of the present study were as follows:

1) Body weight, white adipose tissue weight and liver weight: HFD-fed mice had significantly higher body weight ($P<0.05$) and WAT amount ($P<0.05$) than control diet-fed mice. PHF group had significantly lower body weight gain (15.9% less, $P<0.05$) and less WAT (19.9% less, $P<0.05$) than SHF group. Less WAT was also observed in PC group than SC group (29.7% less, $P<0.05$). Liver weight was significantly lower in PNO-fed mice in comparison to SBO-fed mice ($P<0.05$).

2) Serum and liver lipid levels: PNO-fed mice had significantly higher NEFA than SBO-fed mice (P<0.05). Whereas serum and liver TG concentrations were not affected by either fat amount or oil type.

3) Expression of genes involved in intestinal FA uptake as well as chylomicron assembly: PNO-fed mice had significantly lower *Cd36* mRNA levels (P<0.05) and tendency of lower *ApoA4* mRNA levels (P=0.07) than SBO-fed mice in intestine. Besides, PHF group showed a significant lower *ApoA4* gene expression than SHF group (P<0.05).

4) Expression of genes involved in hepatic lipid uptake and channeling: PNO-fed mice had significantly higher mRNA expression of *Lrp1* and *Fatp5* than SBO-fed mice in liver (P<0.05)

5) Expression of genes involved in hepatic TG lipolysis and FA oxidation: PNO-fed mice had a tendency of higher *Atgl* mRNA levels (P=0.08), and significantly higher *Cpt1a* and Acadl mRNA levels in liver (P<0.05). Mice in PC group had significantly higher *Acadl* mRNA levels compared to mice in SC group (1.2-fold, P<0.05).

6) Expression of genes involved in hepatic TG synthesis and VLDL assembly: PNO consumption did not exert any influence on *Dgat2* mRNA level. *ApoB100* mRNA expression was significantly up-regulated in PNO-fed mice compared with those fed SBO (P<0.05).

The results indicate that PNO-fed groups had significantly lower weight gain and less amount of white adipose tissue compared to SBO-fed groups. The lower *Cd36* mRNA expression and a tendency of lower *ApoA4* mRNA level in PNO consumption groups suggest that PNO may decrease activities of intestinal FA uptake and chylomicron assembly in intestine. The tendency of higher *Atgl* mRNA expression, together with the significantly higher *Cpt1a*, *Acadl* and *ApoB100* mRNA levels in PNO-fed group may imply that PNO could increase hepatic TG lipolysis; mitochondrial FA oxidation and VLDL assembly.

In conclusion, PNO replacement might function to prevent excessive lipid absorption and chylomicron secretion into body circulation from intestine in HFD fed mice. It also could enhance hepatic lipid metabolism in both control and HFD fed mice. Overall, this study may indicate PNO as potential dietary supplement for preventing metabolic dysregulations of lipids in intestine and liver seen with obesity.

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국문초록

고지방 식이로 유도된 비만 마우스에서 잣기름이 소장과 간 지방 대사에 미치는 영향

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잣기름의 섭취는 체중 증가를 감소시키고 고지방 식이로 유도한 비만 마우스에서의 간지방증을 예방하는 데에 긍정적인 영향이 있다고 보고되어 왔다. 본 연구에서는 고지방식이 중 일부를 잣기름으로 대체하였을 때 잣기름이 소장과 간에서의 지질 대사에 미치는 영향을 보고자 하였다. 5 주령의 수컷 C57BL/6 마우스를 네 군으로 나눈 후 네 가지 실험 식이를 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 15% 라아드로, 30%는 콩기름이나 잣기름으로 공급하는 고지방 식이 (SHF 또는 PHF)로 구성되었다. 장내 지방산 흡수 와 운반(*Cd36*, *Fatp4*, *Acsl5*, *Acbp*), 카일로미크론 합성 (*Mttp*,

ApoB48, *ApoA4*), 간 지질의 흡수와 운반 (*Lrp1*, *Fatp5*, *Acsl1*, *Acbp*), 간 중성 지방 분해와 지방산 산화 (*Atgl*, *Cpt1a*, *Acadl*, *Ehhadh*, *Acaa1*)와 초저밀도 지단백 합성 (*ApoB100*) 관련 유전자의 mRNA 발현량을 Real-time PCR 로 측정하였다. 전반적으로 잣기름 섭취군에서 체중 증가량 ($P<0.05$)과 백색 지방량 ($P<0.05$)이 적었다. 소장에서 잣기름 섭취군의 *Cd36* 의 발현량은 유의적으로 낮았고($P<0.05$), *ApoA4* 의 발현량은 SHF 군보다 PHF 군이 유의적으로 낮았다 ($P<0.05$). 또한 간 지질 대사 지표의 결과를 보면 *Atgl* 과 *Cpt1a* 의 발현량은 잣기름 섭취군이 높은 경향성을 보였다 (*Atgl*, $P=0.08$; *Cpt1a*, $P=0.05$). *Acadl* 및 *ApoB100* 의 발현량은 잣기름 섭취군에서 유의적으로 높았다 ($P<0.05$). 잣기름 섭취군에서 *Cd36* 과 *ApoA4* 이 모두 낮은 발현량을 보인 것으로 보아 잣기름이 소장에서 지방 흡수와 카일로미크론 합성을 감소시킬 수 있다고 할 수 있다. 또한 잣기름 섭취군에서 *Atgl* 과 *Cpt1a* 의 발현량이 높은 경향이 있고 *Acadl* 및 *ApoB100* 의 발현량이 유의하게 높았다는 것으로 보아 잣기름 섭취가 간에서의 중성 지방 분해를 증가시키며 지방산 산화 및 VLDL 합성은 촉진할 수 있다는 가능성을 제시한다. 결론적으로 본 연구는 고지방 식이를 섭취한 마우스에서 잣기름이 과도한 장 지방 흡수하는 것을 방지할 뿐만 아니라 간 지질 대사를 개선하는 가능성도 있음을 시사한다.

주요어: 잣기름, 고지방 식이, 소장, 간, 지방 대사

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