Ethanol Extract of Persimmon Tree Leaves Improves Blood Circulation and Lipid Metabolism in Rats Fed a High-Fat Diet

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ABSTRACT The leaves of the persimmon tree (PL) are known to have beneficial effects on hyperglycemia, dyslipidemia, and nonalcoholic fatty liver disease. We recently demonstrated that PL had antithrombotic properties *in vitro*. However, little is known about the antiplatelet and anticoagulant properties of PL *in vivo*. Omega-3 fatty acid (n-3 FA)-containing fish oil has been widely prescribed to improve blood circulation. This study compared the effects of dietary supplementation with an ethanol extract of PL or n-3 FA on blood coagulation, platelet activation, and lipid levels *in vivo*. Sprague–Dawley rats were fed a high-fat diet with either PL ethanol extract (0.5% w/w) or n-3 FA (2.5% w/w) for 9 weeks. Coagulation was examined by monitoring the activated partial thromboplastin time (aPTT) and prothrombin time. We examined plasma thromboxane B₂ (TXB₂), serotonin, and soluble P-selectin (sP-selectin) levels. The aPTT was significantly prolonged in the PL and n-3 FA supplement groups. PL also attenuated the TXB₂ level and lowered arterial serotonin transporter mRNA expression, although it did not alter plasma serotonin or sP-selectin levels. C-reactive protein and leptin levels were significantly reduced by PL and n-3 FA supplementation. In addition, PL decreased plasma total- and low-density lipoprotein-cholesterol levels, as did n-3 FA treatment. These results indicated that the PL ethanol extract may have the potential to improve circulation by inhibiting blood coagulation and platelet activation and by reducing plasma cholesterol levels.

KEY WORDS: • blood circulation • coagulation • lipid metabolism • persimmon tree leaves • platelet activation

INTRODUCTION

THE GLOBAL PREVALENCE OF obesity has increased over recent decades.^{1,2} Obesity is associated with metabolic syndrome, which increases the risk for cardiovascular diseases such as atherosclerosis, thrombotic complications, and myocardial infarction.³ An increased level of adipose tissue elicits pathophysiologic processes involved in inflammation, insulin resistance, and dyslipidemia.⁴ Furthermore, obesity is characterized by multiple hemostatic disturbances, including enhanced platelet activation.⁵ Obesity-induced hyperlipidemia and inflammation contribute to elevated plaque formation⁶ because biologically active molecules produced by adipose tissue, such as some adipokines and free fatty acids, disturb the hemostatic fibrinolytic system and platelet function.³ Obese subjects have increased plasma levels of procoagulant factors, and their risk for a cardiovascular

events is enhanced by this hypercoagulable state.⁷ Indeed, circulating levels of specific coagulation factors (VIII, IX, XI, and XII) are increased in nonalcoholic fatty liver disease independent of obesity, suggesting that the fatty liver can increase the risk of thrombosis.⁸

Regulation of hemostasis is an important process, maintaining the integrity of granules in the blood vessel system.⁹ Thrombosis is normally triggered by extravasation of blood, which induces signals that result in the recruitment of circulating platelets to the injured sites and subsequent blood coagulation.¹⁰ A pathological occlusive thrombosis can occur when hyperactivation of platelets or other blood coagulating factors causes excess blood clotting.^{11,12} Platelet activation is controlled by a complex signaling cascade involving many signaling proteins (*e.g.*, thromboxane A₂ [TXA₂], P-selectin, serotonin, and prostacyclin [PGI₂]).¹³ After platelets are stimulated by vascular injury, their shape can be altered, thrombin synthesis is increased, and the contents of their intracellular granules are released.¹⁴ Dense granules contain small molecules such as serotonin, adenosine diphosphate (ADP), and adenosine triphosphate, while α -granules contain protein mediators, including P-selectin,

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fibrinogen, and Von Willebrand factor.¹⁵ Platelet activation and blood coagulation are mutually dependent and complementary in their interactive processes of hemostasis and thrombosis.¹⁶

Persimmon tree (*Diospyros kaki*) leaves (PL) have long been used to make a tea that is consumed in Korea. They contain a variety of phytochemicals such as catechin, and quercetin, which act as antioxidants.^{17,18} Our previous study showed that PL supplementation improved hyperglycemia, dyslipidemia, and hepatic fat accumulation in type 2 diabetic *db/db* mice.¹⁹ Treatment with PL extract decreased the blood viscosity, plasma viscosity, and fibrinogen level, and stimulated blood circulation.²⁰ Miao *et al.* have recently suggested that the flavonoids present in PL can inhibit cerebrovasculature, improve blood supply to the brain, and relieve ischemia-induced pathological damage in mice.²¹ Moreover, a purified PL fraction had anticoagulant activity, produced by inhibiting thrombin *in vitro*.²² However, little is known about the antithrombotic properties of PL extracts *in vivo*.

Accordingly, we investigated the effects of PL extracts on blood coagulation, platelet activation, and lipid levels in rats fed high-fat diets (HFD), which causes increased platelet aggregation and thrombus formation.^{23,24} In previous *in vivo* studies, the doses of powdered PL and ethanol extract of PL were 5% (5 g/100 g diet, w/w) and 0.7% (0.7 g/100 g diet, w/w), respectively.^{19,25} In this study, 0.5% (w/w) of PL ethanol extract dose was chosen to investigate its physiological activities at a lower dose than 0.7%.

The effects of PL were compared with those of omega-3 fatty acids (n-3 FA), which not only inhibit platelet function by reducing collagen-induced platelet aggregation and thromboxane B₂ (TXB₂) release, but also improve inflammatory signaling pathways and decrease plasma triglyceride levels.^{25,26}

MATERIALS AND METHODS

Preparation of PL extracts

PL extract was provided by CJ CheilJedang, Inc. (Seoul, Korea). PL were collected from air-dried whole plants, harvested in the Kyungsangdo area of Korea. The leaves were extracted with 50% ethanol and concentrated under reduced pressure. After the drying process, the ethanol extract yield from the leaf was 3.58 kg of powdered material from 20.0 kg leaves (17.9%). The quantities of the three major PL flavonoids (catechin, epicatechin, and epicatechin gallate) present in the dried ethanol extract were determined by high-performance liquid chromatography on an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA, USA) using a Venusil XBP C18 column (4.6 × 250 mm, film thickness: 5 μm; Bonna-Agela Technologies, Wilmington, DE, USA) and a two-mobile-phase gradient elution system. Mobile phase A contained acetonitrile, ethyl acetate, and 0.1% aqueous phosphoric acid (8.5:2:89.5, v/v/v), and mobile phase B contained 50% aqueous acetonitrile. The flow rate was set at 0.5 mL/min with an injection volume of 10 μL, and ultraviolet detection was performed at 280 nm.

According to these measurements, the dried ethanol PL extract contained 1.3 mg/g of catechin, 2.0 mg/g of epicatechin, and 1.4 mg/g of epicatechin gallate.

Animals and diets

Five-week-old male Sprague–Dawley (SD) rats were purchased from the Central Laboratory (Seoul, Korea). The animals were individually housed at a constant temperature (24°C) under a 12-h light/dark cycle and were fed a pelletized commercial nonpurified diet for 2 weeks after arrival. The rats were then randomly divided into three groups (*n* = 10 per group) and fed with the following diets for 9 weeks: the HFD controls received a 20%-fat diet based on the AIN-76 diet, plus 1% cholesterol, w/w; the PL extract group received this HFD, supplemented with 0.5% PL extract, w/w; and the n-3 FA positive control group received n-3 FA diet (EPAX, Ålesund, Norway), which was a HFD supplemented with 2.5% n-3 FA, w/w. Diet compositions are presented in Table 1. The rats had free access to food and water during the experimental period. Their food intake and body weight were measured daily and weekly, respectively.

At the ninth week, rats were fasted for 12 h, anesthetized with diethyl ether, and sacrificed. Blood was taken from the inferior vena cava and then centrifuged (Mega 17R, Hanil Science Industrial, Incheon, Korea) at 1000 g for 15 min at 4°C to obtain plasma, which was used to analyze plasma biomarkers. After blood collection, the liver, adipose tissues, and artery were promptly removed, rinsed, weighed, frozen in liquid nitrogen, and stored at –70°C. This animal study protocol was approved by the Ethics Committee for animal studies at Kyungpook National University, Republic of Korea (KNU 2012-135).

Measurement of anticoagulant activity

The anticoagulant activity was examined by monitoring the activated partial thromboplastin time (aPTT) and the prothrombin time (PT). aPTT and PT were determined using

TABLE 1. DIET COMPOSITION FOR ANIMAL EXPERIMENT

<i>Ingredient (g)</i>	<i>HFD</i>	<i>n-3 FA</i>	<i>PL</i>
Casein	200.00	200.00	200.00
D,L-methionine	3.00	3.00	3.00
Corn starch	111.00	111.00	111.00
Sucrose	370.00	370.00	365.00
Cellulose powder	50.00	50.00	50.00
Corn oil	30.00	30.00	30.00
Lard	170.00	145.00	170.00
Mineral mixture (AIN-76)	42.00	42.00	42.00
Vitamin mix (AIN-76)	12.00	12.00	12.00
Choline bitartrate	2.00	2.00	2.00
Cholesterol	10.00	10.00	10.00
tert-Butylhydroquinone	0.04	0.04	0.04
Persimmon leaf ethanol extract	—	—	5.00
Omega-3 (60% EPA and DHA)	—	25.00	—
Total (g)	1000.04	1000.04	1000.04

HFD, high-fat diet (20% fat, 1% cholesterol, w/w); n-3 FA, HFD + 2.5% omega-3 fatty acid, w/w; PL, HFD + 0.5% persimmon leaf, w/w.

a Thrombotimer (Behnk Elektronik, Norderstedt, Germany), according to the manufacturer's instructions. Briefly, plasma (100 μ L) was incubated for 3 min at 37°C. aPTT reagent (100 μ L) was added to the mixture and incubated for 3 min at 37°C. Thereafter, the clotting time was recorded after adding 20 μ M CaCl₂ (100 μ L). For the PT assay, plasma (100 μ L) was incubated for 3 min at 37°C, and the clotting time was recorded after adding PT reagent.

Plasma platelet activation maker levels

To measure plasma levels of platelet activation makers, commercial enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, San Diego, CA, USA) were used to detect serotonin, TXB₂, and soluble P-selectin (sP-selectin).

Plasma adipocytokine levels

Quantitative ELISA kits were used to determine plasma adiponectin and leptin levels (R&D Systems, Minneapolis, MN, USA). The levels of plasma cytokines (interleukin [IL]-10 and C-reactive protein [CRP], and tumor necrosis factor alpha [TNF- α]) were determined using multiplex detection kits from Bio-Rad (Hercules, CA, USA). All samples were assayed in duplicate and analyzed with a Luminex 200 LabMAP system (Luminex, Austin, TX). Data analyses were carried out using Bio-Plex Manager software version 4.1.1 (Bio-Rad, Hercules, CA, USA).

Plasma and hepatic lipid levels

The following plasma lipid concentrations were determined using commercially available kits: total cholesterol, triglyceride, high-density lipoprotein (HDL)-cholesterol (Asan, Seoul, Korea), low-density lipoprotein (LDL)-cholesterol (MyBioSource) free FA, and phospholipids (Wako Chemicals, Richmond, VA, USA). The atherogenic index (AI) was calculated using the following formula: $AI = ([\text{Total cholesterol}] - [\text{HDL-cholesterol}]) / [\text{HDL-cholesterol}]$. The ratio of HDL-cholesterol to total cholesterol (HTR) was calculated using the following formula: $[\text{HDL-cholesterol} / \text{Total cholesterol}]$. Hepatic lipids were extracted according to the method by Folch *et al.*,²⁷ and the dried lipid residues were dissolved in 1 mL of ethanol. Triton X-100 and a sodium cholate solution prepared in distilled water were added to 200 μ L of the dissolved lipid solution for emulsification. The hepatic triglyceride, cholesterol, and FA levels were analyzed using the same commercially available kits as those used for the plasma analyses.

Lipid-regulating enzyme activities

To measure the activities of lipid-regulating enzymes in the liver, samples were prepared and analyzed according to the methods described by Kim *et al.*²⁸ Briefly, fatty acid synthase (FAS) activity was determined using a spectrophotometric assay according to the method described by Carl *et al.*,²⁹ where one unit of FAS activity oxidized 1 nmol of nicotinamide adenine dinucleotide phosphate (NADPH) per minute at 30°C. Carnitine palmitoyl-CoA transferase

(CPT) activity was determined according to the method described by Markwell *et al.*,³⁰ Glucose-6-phosphate dehydrogenase (G6PD) activity was determined according to the method described by Pitkanen *et al.*³¹ Malic enzyme (ME) activity was determined according to the method by Ochoa³² and the results were expressed as nmol/min per mg protein. Protein concentration was measured by the Bradford method by using bovine serum albumin standards.³³ Phosphatidate phosphohydrolase (PAP) activity was determined using the method described by Walton and Possmayer.³⁴

RNA extraction

Total RNA was isolated from the artery using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA), according to the manufacturer's instructions. RNase-free water was added and RNA was quantified using NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) prior to storage at -70°C .

Real-time quantitative polymerase chain reaction analysis

Total RNA (1 μ g) was reverse transcribed to cDNA by using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). mRNA expression was then quantified by real-time quantitative polymerase chain reaction using the QuantiTect SYBR Green PCR kit (Qiagen) on the CFX96TM RT-PCR system (Bio-Rad, Hercules, CA, USA). Gene-specific primers were used to amplify serotonin transporter (*SERT*), cyclooxygenase 2 (*COX2*), and selectin P (*SELP*). The sequences of the primers were as follows: *SERT*, 5'-CACAGCGGACAG GGCAGAGC-3' (forward), 5'-GGAGGCGATGTAAAAGG CGATGAT-3' (reverse); *COX2*, 5'-ACTTGCTCACTTTGT TGAGTCATTC-3' (forward), 5'-TTTGATTAGTACTGTA GGGTTAATG-3' (reverse); *SELP*, 5'-ACAAGGCAGCTTG GACTGCTCT-3' (forward), 5'-TGTTACACCTTTGCAGG TTGGTGG-3' (reverse). Cycle thresholds (C_t) were determined based on SYBR green emission intensity during the exponential phase. C_t data were normalized using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was stably expressed across all experimental groups. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.³⁵

Histological analysis

The liver and epididymal white adipose tissue were fixed in 10% formalin buffer solution and then routinely processed for paraffin embedding. The 4- μ m sections of each tissue were stained with hematoxylin and eosin and observed under an optical microscope (Zeiss Axioscope, Jena, Germany) with magnifying powers of $\times 200$ and $\times 400$, respectively.

Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean. Significant differences among the groups were determined using one-way analysis of variance in SPSS (version 18; SPSS, Inc., Chicago, IL, USA). Duncan's multiple-range test was performed if differences were considered to be statistically significant, when $P < .05$.

TABLE 2. EFFECT OF DIETARY SUPPLEMENTATION OF OMEGA-3 AND PERSIMMON LEAF ON BODY WEIGHT, BODY WEIGHT GAIN, AND FOOD INTAKE IN RATS FED HIGH-FAT DIET FOR 9 WEEKS

	HFD	n-3 FA	PL
Initial body weight (g)	185.67 ± 2.63	181.85 ± 4.49	172.93 ± 10.61
Final body weight (g)	531.93 ± 12.39	529.01 ± 14.21	532.34 ± 12.14
Body weight gain (g/day)	5.78 ± 0.23	5.93 ± 0.21	6.03 ± 0.19
Food intake (g/day)	19.15 ± 0.64	20.01 ± 0.88	19.24 ± 0.73

RESULTS

Food intake, body weight, and tissue morphologies

Supplementation with PL extract did not alter final body weight or body weight gain in rats fed a HFD (Table 2). There were no significant differences between the food intakes in the three study groups over the 9-weeks feeding period (Table 2). However, histological analyses revealed that rats supplemented with PL extract or n-3 FA exhibited smaller epididymal adipocyte size and less accumulation of hepatic lipid droplets, as compared with the HFD control group (Fig. 1).

Blood coagulation factors

To determine whether supplementation with PL extract or n-3 FA had anticoagulant effects, PT and aPTT were evaluated. The n-3 FA positive control group showed significantly prolonged PT and aPTT, as compared with the HFD control group. However, compared with the HFD group, PL extract group showed significantly prolonged aPTT but not PT (Table 3).

Plasma levels of platelet activation markers

Supplementation with n-3 FA significantly decreased plasma TXB₂ and sP-selectin levels, as compared to the

TABLE 3. EFFECT OF DIETARY SUPPLEMENTATION OF PERSIMMON LEAF AND OMEGA-3 ON ANTICOAGULANT ACTIVITY BASED ON PROTHROMBIN TIME AND ACTIVATED PARTIAL THROMBOPLASTIN TIME IN RATS FED HIGH-FAT DIET FOR 9 WEEKS

	HFD	n-3 FA	PL
PT	12.98 ± 0.16 ^b	14.70 ± 0.85 ^a	13.80 ± 0.36 ^{ab}
aPTT	11.92 ± 0.58 ^b	14.78 ± 0.86 ^a	16.33 ± 0.42 ^a

Data are mean ± SE.

Means not sharing a common superscript lowercase letter are significantly different among the HFD fed groups at $P < .05$.

aPTT, activated partial thromboplastin time; PT, prothrombin time.

HFD group. PL extract significantly reduced the plasma TXB₂ level in comparison to the HFD group, without altering plasma serotonin or sP-selectin levels (Fig. 2).

Plasma adipokine and cytokine levels

Both dietary PL extract and n-3 FA significantly reduced the plasma leptin level in HFD-fed rats, compared with the control group. Although plasma adiponectin level was not significantly changed, the leptin:adiponectin (L:A) ratio was significantly reduced in the PL group (Fig. 3). In addition, both PL extract and n-3 FA supplements significantly elevated the plasma IL-10 level in comparison to that observed in the control group and lowered plasma CRP levels (Fig. 3). No statistically significant differences in plasma TNF- α level were observed, although the levels tended to be lower in animals receiving PL or n-3 FA supplements (Fig. 3).

Plasma and hepatic lipid levels

Compared to the HFD control group, the PL extract group showed significantly lower plasma total cholesterol and apolipoprotein B (Apo B) concentrations (Table 4). PL extract also tended to decrease the non-HDL cholesterol and HTR. However, no significant differences were observed

FIG. 1. Effect of dietary supplementation of omega-3 and persimmon leaf on hepatic and white adipose tissue morphology in rats fed HFD for 9 weeks. Representative photomicrographs of liver and epididymal white adipose tissue are shown at $\times 200$, $\times 400$ magnification, respectively. HFD, high-fat diet (20% fat, 1% cholesterol, w/w); n-3 FA, HFD + 2.5% omega-3 fatty acid, w/w; PL, HFD + 0.5% persimmon leaf, w/w. Color images available online at www.liebertpub.com/jmf

FIG. 2. Effect of dietary supplementation of omega-3 and persimmon leaf on plasma TXB₂, serotonin, and sP-selectin levels in rats fed HFD for 9 weeks. Data are expressed as the mean $\pm$ SE. Means not sharing a common lowercase letter (a, b) are significantly different among the groups at $P < .05$. sP-selectin, soluble P-selectin; TXB₂, thromboxane B₂.

between the study group levels of plasma triglyceride, HDL-cholesterol, or free FA. The PL extract and n-3 FA decreased AI. Supplementation with n-3 FA, a positive control, significantly decreased plasma total cholesterol, non-HDL cholesterol, free FA, phospholipid, Apo A, and Apo B levels compared with those in the control group, while it increased HTR. Measurement of hepatic lipid levels indicated that the PL extract and the n-3 FA supplement significantly lowered the hepatic triglyceride level, compared with those observed in the HFD control group (Table 4).

Liver lipid-regulating enzyme activities

Among the hepatic lipogenic enzymes, activities of FAS, G6PD, and ME were significantly lower in both the PL group and the n-3 FA group, compared with the HFD control group. However, significantly reduced hepatic CPT and PAP activities were observed in rats supplemented with PL extract, but not in those supplemented with n-3 FA (Table 5).

Arterial expression of genes involved in platelet activation

In arterial tissue, mRNA expression of *SERT* was significantly lower in the PL group, compared with the HFD group. *COX2* and *SELP* mRNA expression was significantly lower in the n-3 FA group only (Fig. 4).

DISCUSSION

Several *in vitro* studies have suggested that PL has potentially beneficial effects on vascular thrombotic risk and on blood circulation. However, no published reports have described the antithrombotic effects of PL *in vivo*. Therefore, this study demonstrated for the first time that dietary supplementation using a PL extract could improve blood circulation by inhibiting coagulation and platelet activation and reducing plasma cholesterol levels in SD rats fed a HFD for 9 weeks.

Increased blood coagulation can be harmful because it increases the risk for thrombosis.^{36,37} The aPTT is commonly

FIG. 3. Effect of dietary supplementation of omega-3 and persimmon leaf on plasma adipocytokine levels in rats fed HFD for 9 weeks. Data are expressed as the mean $\pm$ SE. Means not sharing a common lowercase letter (a–c) are significantly different among the groups at $P < .05$. CRP, C-reactive protein; IL-10, interleukin 10; L:A ratio, leptin:adiponectin ratio; TNF- α , tumor necrosis factor alpha.

TABLE 4. EFFECT OF DIETARY SUPPLEMENTATION OF OMEGA-3 AND PERSIMMON LEAF ON PLASMA LIPID PROFILES IN RATS FED HIGH-FAT DIET FOR 9 WEEKS

	HFD	n-3 FA	PL
Plasma			
Triglyceride (mmol/L)	1.20 ± 0.10	0.95 ± 0.09	1.01 ± 0.07
Total-C (mmol/L)	2.88 ± 0.30 ^b	1.89 ± 0.16 ^a	2.26 ± 0.07 ^a
HDL-C (mmol/L)	0.37 ± 0.02	0.37 ± 0.19	0.38 ± 0.03
Non HDL-C (mmol/L)	2.70 ± 0.60 ^b	1.55 ± 1.05 ^a	1.96 ± 0.05 ^{ab}
LDL-C (mmol/L)	2.29 ± 0.56 ^b	1.11 ± 0.17 ^a	1.45 ± 0.14 ^a
Free fatty acid (mmol/L)	0.80 ± 0.04 ^b	0.57 ± 0.05 ^a	0.75 ± 0.03 ^b
Phospholipid (mmol/L)	1.52 ± 0.13 ^b	1.19 ± 0.06 ^a	1.43 ± 0.03 ^b
Apolipoprotein A-I (mg/dL)	8.37 ± 0.40 ^b	7.48 ± 0.31 ^a	8.02 ± 0.31 ^{ab}
Apo B (mg/dL)	10.34 ± 0.35 ^b	8.20 ± 0.36 ^a	8.39 ± 0.49 ^a
HTR (%)	13.93 ± 2.18 ^b	20.15 ± 1.73 ^a	14.92 ± 0.48 ^{ab}
AI	7.38 ± 1.79 ^b	4.03 ± 0.42 ^a	5.47 ± 0.79 ^a
Apo A-I/Apo B	0.84 ± 0.04	0.94 ± 0.06	1.01 ± 0.05
Liver			
Triglyceride (mmol/g liver)	6.44 ± 0.42 ^b	4.00 ± 0.42 ^a	5.03 ± 0.39 ^a
Cholesterol (mmol/g liver)	3.80 ± 0.26	3.19 ± 0.85	3.45 ± 0.57
Fatty acid (mmol/g liver)	4.92 ± 0.48	4.91 ± 0.59	4.48 ± 0.42

Data are mean ± SE.

Means not sharing a common superscript lowercase letter are significantly different among the HFD-fed groups at $P < .05$.

AI, atherogenic index; Apo B, apolipoprotein B; HDL-C, high-density lipoprotein-cholesterol; HTR, ratio of HDL-cholesterol to total cholesterol; LDL-C, low-density lipoprotein-cholesterol.

TABLE 5. EFFECT OF DIETARY SUPPLEMENTATION OF OMEGA-3 AND PERSIMMON LEAF ON THE HEPATIC LIPID-REGULATING ENZYME ACTIVITIES IN RATS FED HIGH-FAT DIET FOR 9 WEEKS

	HFD	n-3 FA	PL
FAS (μ mol oxidized/min/mg protein)	81.22 ± 17.05 ^b	26.00 ± 3.40 ^a	46.27 ± 8.59 ^a
G6PD (nmol/min/mg protein)	145.06 ± 13.04 ^c	81.05 ± 12.13 ^a	112.07 ± 10.35 ^b
ME (nmol/min/mg protein)	167.44 ± 33.44 ^b	99.05 ± 7.87 ^a	99.80 ± 5.04 ^a
CPT (nmol/min/mg protein)	20.61 ± 3.24 ^b	24.24 ± 2.02 ^b	10.49 ± 2.40 ^a
PAP (μ mol oxidized/min/mg protein)	0.51 ± 0.02 ^b	0.49 ± 0.01 ^b	0.40 ± 0.02 ^a

Data are mean ± SE.

Means not sharing a common superscript lowercase letter are significantly different among the HFD-fed groups at $P < .05$.

CPT, carnitine palmitoyl-CoA transferase; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; ME, malic enzyme; PAP, phosphatidate phosphohydrolase.

used for the detection of coagulation factor deficiencies,³⁸ and a lower aPTT is associated with increased risk of thrombosis.^{39,40} Moreover, aPTT has been found to decrease with increased body mass index (BMI)⁴¹ and BMI was suggested to represent a significant independent predictor of the activities of intrinsic clotting factors such as factors IX, XI, and XII.⁴² In this study, we found that aPTT was prolonged in high-fat fed rats supplemented with n-3 FA and PL, which indicated that the n-3 FA and PL extract could play a beneficial regulatory role in the coagulation process by inhibiting the intrinsic coagulation pathway. Increased platelet reactivity is reported to play a major role in elevating the risk of atherothrombosis.⁴³ In addition, it is known that plasma TXA₂ and P-selectin levels are increased by arachidonic acid production in metabolic syndrome.^{44,45} Supplementation of PL extract significantly decreased the plasma level of TXB₂, a marker of platelet activation, although plasma serotonin and sP-selectin levels were not changed. Supplementation of n-3 FA, positive control, significantly decreased the plasma level of TXB₂ and sP-selectin. Inhibition of SERT can decrease platelet 5-HT content, inhibit thrombosis, and exacerbate bleeding.⁴⁶ We found that the PL extract significantly downregulated mRNA expression of arterial *SERT*, which is involved in platelet activation. *COX2* expression plays a role in TXB₂ synthesis and platelet aggregation.⁴⁷ In this study, although supplementation with the PL extract tended to downregulate mRNA expression of arterial *COX2*, the plasma level of TXB₂ was reduced. Accordingly, a decrease in plasma TXB₂ may partially be explained by the potential reduction in *COX2* mRNA expression. Arterial *SELP* mRNA expression was significantly downregulated by n-3 FA supplementation, similar to the plasma sP-selectin level.

There is robust evidence that increased plasma total cholesterol and LDL-cholesterol levels are directly related to the development of atherosclerotic plaque and cardiovascular disease.^{6,48} In addition, LDL-cholesterol enhances platelet activation by inhibiting the Na⁺/K⁺ antiporter.⁴⁹ Apo B is also a useful predictor of vascular atherosclerotic disease.⁵⁰ In this study, supplementation of both n-3 FA and PL extract effectively reduced the plasma total cholesterol and LDL-cholesterol, and the Apo B levels. AI was also significantly lowered by PL extract supplementation, although the plasma HDL-cholesterol level was not significantly increased. Accordingly, these results indicate that the effect of dietary supplementation of n-3 FA and PL on plasma lipid levels are similar. The effect of PL extract on the plasma HDL-cholesterol level in HFD-fed rats was not consistent with the findings of a previous study¹⁹ where PL decreased plasma triglyceride and total cholesterol levels, while increasing plasma HDL-cholesterol in type 2 diabetic *db/db* mice. This discrepancy could be due to differences in the animal models employed in these two studies.

In this study, we observed that n-3 FA and PL supplementation significantly reduced liver triglyceride content and lipid droplet accumulation. The improvement of hepatic steatosis observed in the n-3 FA and PL group was partly associated with marked decreases in the activity of hepatic

FIG. 4. Effect of dietary supplementation of omega-3 and persimmon leaf on artery mRNA expression related to platelet activation in rats fed HFD for 9 weeks. Data are expressed as the mean $\pm$ SE. Means not sharing a common lowercase letter (a, b) are significantly different among the groups at $P < .05$. COX2, cyclooxygenase 2; SELP, selectin P; SERT, serotonin transporter.

FAS, a key enzyme involved in *de novo* FA biosynthesis. We also found that the PL extract supplement led to decreased activity of hepatic CPT, a major rate-limiting enzyme in mitochondrial FA uptake and oxidation. Since FAS inhibition leads to an increase in hepatic malonyl-CoA, a potent inhibitor of CPT,^{51,52} this PL-mediated decrease in hepatic CPT activity could be a secondary consequence of decreased hepatic FAS activity, although we did not determine hepatic malonyl-CoA level in this study. However, there was no significant difference in hepatic CPT activity in the n-3 FA group. The activities of other adipogenic enzymes, G6PD and ME, were also significantly lowered by PL extract supplementation. Moreover, PL extract supplementation effectively reduced the activity of PAP, an enzyme catalyzing liver triglyceride synthesis. Taken together, our observations suggested that the effects of the PL extract on the activities of lipid-regulating enzymes were consistent with the decrease in hepatic triglyceride levels.

Under high fat-fed conditions, adipose tissue actively influences the blood coagulation cascade and fibrinolysis. Accordingly, it could lead to increased thrombus formation and insoluble fibrin deposition via the activities of several cytokines and adipokines, including leptin and TNF- α .⁴ The leptin receptor is present in platelets⁵³; leptin promotes their aggregation by increasing platelet responses to ADP and thrombin,⁵⁴ and it may accelerate the development of vascular injury.⁵⁵ In addition, the L:A ratio serves as an AI that is superior to using leptin or adiponectin levels alone.⁵⁶ We observed that PL supplementation significantly decreased not only the adipocyte size, plasma leptin level, and the L:A ratio, but also AI in HFD-fed rats. TNF- α stimulates leptin production, reduces adiponectin secretion from adipose tissue, and promotes endothelial adhesion molecule expression,^{57–59} although plasma TNF- α only tended to be decreased by PL extract supplementation. An elevation in CRP is also considered a powerful marker of increased atherothrombotic events^{59,60} and may contribute to thrombosis via several mechanisms.⁵⁶ Additionally, synthesis of CRP is increased by leptin, which acts on the IL-6 receptors in the liver, contributing to the inflammatory state associated with obesity.⁶¹ In this study, n-3 FA and PL extract supplementation reduced the plasma CRP level by 22% and 13.0% compared with the HFD group.

In this study, n-3 FA, positive control, modulated intrinsic, extrinsic coagulation factors, and platelet activation markers, TXB₂, sP-selectin, and plasma lipid levels such as total cholesterol, non-HDL cholesterol, LDL cholesterol, and free FA. Similar to our results, n-3 FA decreased endothelial activation markers, P-selectin, ICAM-1, VCAM-1, and vasoconstrictors such as TXA₂.⁶² Also, n-3 FA prevented thrombosis and atherosclerosis by lowering plasma cholesterol and triglyceride levels and controlling of gene transcription factors involved in lipid metabolism.⁶² Thus, regulation of vascular endothelial function and lipid levels are the mechanisms underlying cardioprotective effects of n-3 FA. Our findings provided considerable evidence that the PL extract may have beneficial effects on blood circulation and cardiovascular disease by similar mechanisms of n-3 FA action in cardiovascular diseases. These beneficial effects of PL may be partly associated with reduced adiposity and improved circulating levels of adipokines.

In conclusion, this study demonstrated for the first time that supplementation of dietary PL extract can ameliorate thrombotic events by inhibiting coagulation factors involved in the intrinsic blood coagulation system and inhibiting platelet activity in rats fed a HFD. These changes were partly associated with the downregulation of *SERT* mRNA expression in the artery. Furthermore, the PL extract attenuated the plasma total and LDL-cholesterol levels and the hepatic triglyceride level, and reducing activities of enzymes involved in the regulation of hepatic FA synthesis. Accordingly, these findings suggested that PL was effective for improving lipid regulation and blood circulation, in a manner similar to n-3 FA, in rats fed a HFD. Thus, this study suggested that PL may be useful for improving blood circulation and reducing cardiovascular disease and hepatic steatosis. As referred to in the Materials section, dried ethanol PL extract contains 1.3 mg/g of catechin, 2.0 mg/g of epicatechin, and 1.4 mg/g of epicatechin gallate equivalent to 3.9 μ M catechin, 6.1 μ M epicatechin, and 3.1 μ M epicatechin gallate. In our previous *in vitro* study, catechin, epicatechin, and epicatechin gallate exerted significant inhibitory activity on blood coagulation and platelet activation at a concentration of 5 μ M.⁶³ Accordingly, these flavonoids in PL possibly played important roles in blood circulation and cardiovascular disease as bioactive components.

However, further studies are needed to clarify the *in vivo* role of these flavonoids in blood coagulation and platelet activation and to determine the optimal human dose of PL.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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