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Alleviation of nonalcoholic fatty liver disease development by genistein in ApoE$^{-/-}$ mice fed a high-fat diet

고지방식이를 섭취한 ApoE$^{-/-}$ 마우스에서 제니스테인에 의한 비알콜성 지방간질환 완화 효과 연구

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Abstract

Alleviation of nonalcoholic fatty liver disease development by genistein in ApoE$^{-/-}$ mice fed a high-fat diet

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Nonalcoholic fatty liver disease (NAFLD) occurs in a wide variety of clinical presentations, ranging from asymptomatic hepatic steatosis to severe nonalcoholic steatohepatitis (NASH). Recent studies reported that ApoE$^{-/-}$ mice fed a high-fat diet (HFD) can be used as a model of NASH as well as arteriosclerosis. Because genistein has been shown to alleviate hepatic steatosis, we investigated the effects of genistein on the development of NASH in ApoE$^{-/-}$ mice fed an HFD. Both wild-type (WT) mice and ApoE$^{-/-}$ mice were fed either an HFD (45% of the calories from fat) or an HFD supplemented with genistein (0.5g/kg diet). After 24 weeks on an HFD, serum triglyceride, total cholesterol, thiobarbituric acid reactive substances, monocyte chemoattractant protein 1 (MCP-1), and alanine aminotransferase

(ALT) levels were more exacerbated in ApoE$^{-/-}$ mice compared to WT mice. In addition, ApoE$^{-/-}$ mice exhibited more severe hepatic fat accumulation and hepatic inflammation. These findings confirmed that ApoE$^{-/-}$ mice fed an HFD are a promising NASH model. Cholesterol levels in both sera and livers were alleviated by genistein in ApoE$^{-/-}$ mice. Furthermore, ApoE$^{-/-}$ mice exhibited a reduced serum MCP-1 and ALT levels as well as hepatic triglyceride and hepatic pro-inflammatory gene expressions such as MCP-1, tumor necrosis factor α, and cyclooxygenase 2 in response to genistein. Hepatic gene expressions related to lipid metabolism including peroxisome proliferator activated receptor gamma (PPARγ), CD36 and monoacylglycerol O-acyltransferase 1 were reduced by genistein in ApoE$^{-/-}$ mice. In particular, serum cholesterol levels were significantly correlated with liver injury and the expression of various hepatic inflammatory genes, indicating that hypercholesterolemia plays an important role in accelerating NASH progression. In summary, genistein acts as an antioxidant resulting in reduced serum lipid peroxidation products and inflammation. Genistein also reduces the expressions of PPARγ and its target genes, resulting in both the influx of the oxidized lipid products into liver and the lipid formation. In conclusion, genistein alleviated metabolic abnormalities including hypercholesterolemia, obesity, and NASH in

ApoE$^{-/-}$ mice fed an HFD.

Key words: genistein, ApoE$^{-/-}$ mice, nonalcoholic fatty liver disease, serum cholesterol, peroxisome proliferator activated receptor gamma

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List of Abbreviations

ABCA1: adenosine triphosphate-binding cassette A1

ALT: alanine aminotransferase

ApoE: apolipoprotein E

COX-2: cyclooxygenase 2

CPT1a: carnitine palmitoyltransferase 1a

CYP7A1: cholesterol 7α-hydroxylase

DGAT1: diacylglycerol acyltransferase 1

FAS: fatty acid synthase

FXR: farnesoid X receptor

HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase

IL-1β: interleukin 1 beta

LDLR: low-density lipoprotein receptor

5-LO: 5-lipoxygenase

LXRα: liver X receptor alpha

MCP-1: monocyte chemoattractant protein 1

MGAT: monoacylglycerol O-acyltransferase 1

NAFLD: nonalcoholic fatty liver disease

NASH: nonalcoholic steatohepatitis

oxLDL: oxidized low-density lipoprotein

PPAR: peroxisome proliferator-activated receptor

RPL19: ribosomal protein L19

RXRα: retinoid X receptor alpha

SCD1: stearoyl-CoA desaturase-1

SREBP: sterol regulatory element-binding protein

TNFα: Tumor necrosis factor alpha

Introduction

Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver damage, ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis), hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis [1]. NAFLD is defined as fat accumulation in the liver exceeding 5% by weight in the presence of < 10g of daily alcohol consumption [2]. With the increasing prevalence of obesity, diabetes, and metabolic syndrome in the general population, NAFLD has become the most common cause of chronic liver disease in western countries and also in other parts of the world including Korea [3-5]. There are existing treatment strategies for NAFLD including weight loss, life-style modifications, insulin-sensitizing agents, and antioxidant therapies, however, there are no licensed medical treatments for NAFLD patients [6].

The 'two hit hypothesis' has become a prevalently accepted framework for understanding the pathogenesis of NAFLD. The first hit mainly consists of lipid accumulation in the liver, a process that is closely linked with insulin resistance. The second hit promotes inflammation, cell death, and fibrosis, which triggers the progression of steatosis to

nonalcoholic steatohepatitis (NASH) [7]. Steatosis itself is generally considered benign and reversible, however, the presence of inflammation can cause further progression of NASH, promoting liver fibrosis, cirrhosis, and eventually liver failure and hepatocellular carcinoma [8]. The etiology of progression from hepatic steatosis to NASH is postulated as a response to lipotoxicity, but the causative lipotoxic molecules have remained unclarified. Current studies have suggested free fatty acids [9], free cholesterol [10], cholesterol-oxidized products [11] and macrophages activated by free cholesterol [12]. Moreover, increased plasma cholesterol levels have been shown to induce the hepatic inflammation in animal models of hyperlipidemia [13-15].

Previous studies have demonstrated that the level of oxidized low-density lipoprotein (oxLDL), the highly reactive form of LDL following lipid peroxidative modification, increases dramatically with hypercholesterolemia both in animal models [16, 17] and in human [18]. OxLDL plays an important role in atherosclerosis [19] and cardiovascular disease [20]. In addition, recently oxLDL has gained attention as a new risk factor for hepatic inflammation. OxLDL as well as 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE can play as ligands for peroxisome proliferator activated receptor gamma (PPARγ) [21]. In addition to two major transcription factors, sterol regulatory element-binding protein

1c (SREBP1c) and carbohydrate responsive element-binding protein, PPARγ is recently recognized as an important transcription factor involved in hepatic steatosis development. It was reported that hepatic PPARγ2 was extremely up-regulated in a murine obesity model, which suggests that PPARγ plays an important role in fatty liver formation [22]. In addition, liver-specific disruption of PPARγ in ob/ob mice improves fatty liver [23].

As shown in **Fig. 1**, PPARγ activated by ligands such as oxLDL can heterodimerize with the retinoid X receptor (RXR) and acts as a transcriptional factor of genes including fat-specific protein 27, 422 adipose P2 protein, CD36, monoacylglycerol O-acyltransferase 1 (MGAT1) and adipose differentiation-related protein [24-26]. Representative scavenger receptors of oxLDL including CD36, scavenger receptor class A, and lectin-like receptor 1 mediate uptake of oxLDL by macrophages and like typical macrophages, Kupffer cells express CD36 [25]. Scavenger receptor-mediated uptake of oxLDL by macrophages transforms macrophages into form cells [27], induces production of numerous proinflammatory cytokines by vascular endothelial cells [28, 29], and mediates monocyte accumulation into the blood vessel walls by inducing MCP-1 expression [30, 31]. Furthermore, the uptake of oxLDL by scavenger receptor in the liver has been shown to be involved in hepatic inflammation [33, 34].

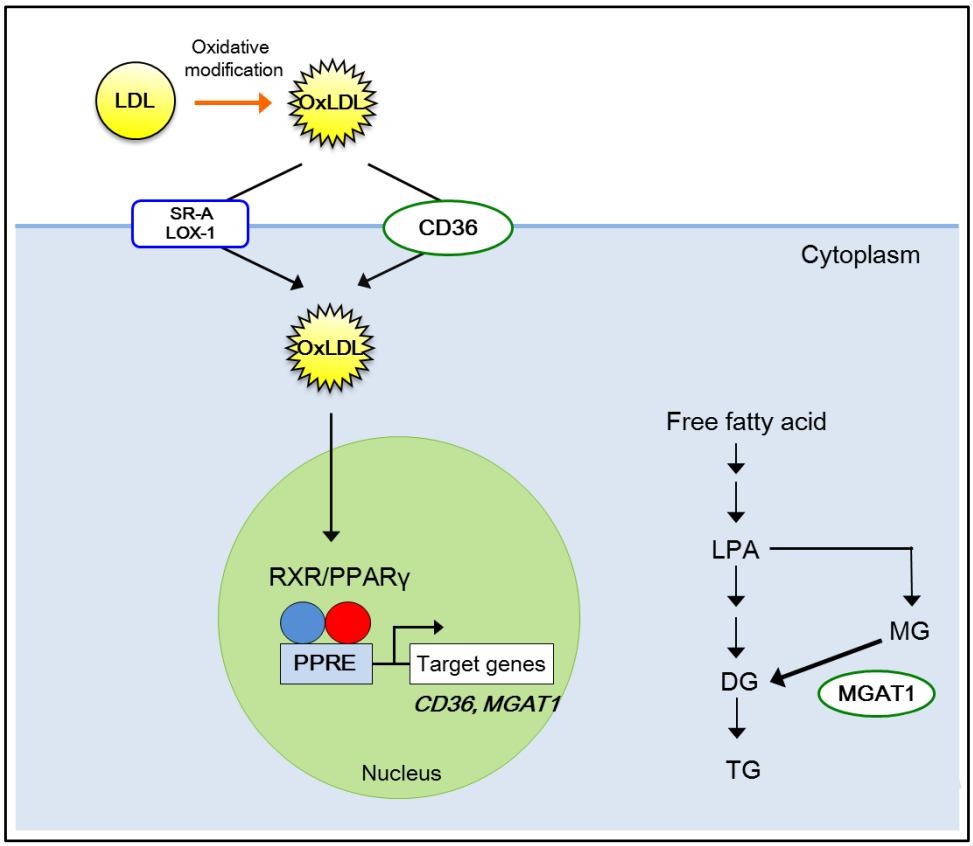


Figure 1. A proposed model for the PPARγ signaling (modified from [32])

DG, diacylglyceride; LOX-1, lectin-like receptor 1; LPA, lysophosphatidic acid; MG, monoacylglyceride, MGAT1, monoacylglycerol O-acyltransferase 1; OxLDL, oxidized low-density lipoprotein; PPAR, peroxisome proliferator activated receptor; PPRE, PPAR-response element; RXR, retinoid X receptor; SR-A, scavenger receptor class A ; TG, triacylglyceride

Apolipoprotein E-deficient mice

Apolipoprotein E (ApoE) is the major protein component of LDL particles and thus involved in hepatic lipoprotein clearance from blood. The absence of this protein promotes severe hypercholesterolemia, subsequently leading to develop lesions of atherosclerosis. Therefore, ApoE$^{-/-}$ mice have been used for studying atherosclerosis. Recent studies have suggested that ApoE$^{-/-}$ mice can be also used as an animal model for studying NASH and the link between atherosclerosis and NASH [13, 33, 34]. The severity of the progression of the diseases can be increased by feeding the mice a high-fat diet [35]. Previous studies have reported that ApoE$^{-/-}$ mice exhibited hyperlipidemia, exacerbated steatosis, increased hepatic oxidative stress, liver injury [44], and increased local inflammatory cytokine production in the liver compared with wild-type mice [38]. It was demonstrated that ApoE$^{-/-}$ mice fed a normal chow diet develop fatty liver since VLDL secretion is severely impaired [36]. The impaired delivery of VLDL from liver to target tissues could partly account for lipid accumulation in the livers of ApoE$^{-/-}$ mice [45].

Genistein

According to recent finding in Japan, prevalence of NASH in women over 50 years of age who are considered as postmenopausal is higher than in men. The decrease of estrogen in postmenopausal women can explain this gender difference [37]. Previous study reported that 17β-estradiol prevents oxidation of LDL as well as foam cell formation [38, 39]. In addition, 17β-estradiol treatment of postmenopausal women is associated with increased LDL resistance to Cu^{2+}-mediated oxidation [40].

Phytoestrogens are plant-derived estrogens that can mimic the actions of 17β-estradiol. The isoflavone genistein is a phytoestrogen found in high concentration in soy and soy products [41]. Genistein has been shown an array of biological activities including attenuating cardiovascular diseases [42] and obesity [43], and acting as an antioxidant [44] and tyrosine protein kinase inhibitor [45]. Additionally, genistein has shown anti-inflammatory activity in vivo [46, 47] and in vitro [48]. Genistein has been shown to prevent hepatic steatosis and inflammation in HFD-induced rats [49, 50]. Likewise, a DNA microarray analysis also showed that genistein regulates hepatic gene expression, especially genes involved in lipid and carbohydrate metabolism in rats fed an HFD [51].

Aim of this study

In this study, we investigated whether dietary genistein would alleviate NASH development in ApoE$^{-/-}$ mice fed an HFD.

Materials and Methods

Experimental animals and diets

During the experiment, mice studies were conducted according to the Animal Care and Use Guidelines from the Institute of Laboratory Animal Resources at Seoul National University (SNU-110524-1). Male wild-type (WT) mice and ApoE$^{-/-}$ mice on a C57BL/6J background were purchased (SLC Inc., Japan) at 6 weeks of age. Mice were initially acclimated with a chow diet for 1 week prior to the start of the study. Subsequently, both WT mice and ApoE$^{-/-}$ mice were divided into two groups and allocated into either a high fat diet (HFD) or an HFD containing 0.05% genistein (LC Laboratories, USA). HFD contained 0.02% cholesterol and 45% calories from fat and the concentration of genistein was 0.5g/kg diet (Feed Lab Co., South Korea). The composition of the diets is given in **Table 1**. Mice were fed with an ad libitum experimental diet and water for 24 weeks and were housed under standard conditions of temperature (23 ± 3°C) and humidity (50 ± 10%), with a 12-hour light/dark cycle. Food intake was measured three times per week and individual body weights were measured weekly over a 24-week period. At the end of the experiments, the mice were fasted for 12 hours and sacrificed. Blood samples were rapidly obtained by cardiac

puncture and centrifuged for 20 min, at 3000 rpm at 4°C. Tissues were removed, washed with phosphate-buffered saline (PBS) and weighed. Serum and tissues were stored at –80°C until analysis. Part of liver was fixed in 10% formalin for histological analysis.

Serum analyses

Serum glucose, triglyceride, total cholesterol, and alanine aminotransferase (ALT) levels were measured by commercial kits and all samples were assayed in duplicate (Asan Pharmaceutical Co., South Korea). Insulin (Millipore, USA) and monocyte chemoattractant protein-1 (MCP-1; R&D Systems, USA) levels were determined using an ELISA kit. The insulin resistance index was estimated by homeostasis model assessment (HOMA-IR) with the following formula: serum glucose × serum insulin/22.5, with serum glucose in mmol/mL and serum insulin in μU/mL.

Table 1. Composition of the experimental diets

Ingredient	High fat diet	High fat diet containing genistein
	(g / kg)	
Cornstarch	84.8	84.8
Casein	233.1	233.0
Maltodextrin 10	116.5	116.5
Sucrose	201.4	201.3
Lard[a]	206.9	206.7
Soybean oil	29.1	29.1
Cellulose	58.3	58.2
Mineral mix	11.7	11.6
Dicalcium phosphate	15.1	15.1
Calcium carbonate	6.4	6.4
Potassium citrate monohydrate	19.2	19.2
Vitamin mix	11.7	11.6
L-Cystine	3.5	3.5
Choline bitartrate	2.3	2.3
Genistein	-	0.5

[a] Typical analysis of cholesterol in lard = 0.95 mg/gram.
Cholesterol (mg)/kg = 196.5.

Hepatic lipid measurements

Hepatic total lipids were extracted according to the method of Folch et al [52]. Briefly, liver tissue was homogenized in PBS (5%, w:v). The protein content of the homogenate was measured using a protein assay kit (Bio-Rad, USA). 300 μl homogenate containing the equal amount of protein (1 mg/ml) was prepared, mixed with 1.2 ml of chloroform/methanol (2:1, v:v), and incubated overnight at 4℃. Thereafter, 240 μL of 0.88% KCl was added for aggregation of non-lipid contents and centrifuged at 1000 × g for 15 min at 4℃. The bottom layer was obtained, aliquoted into 100 μl portions, and evaporated with nitrogen gas. The lipid pellets were resuspended in 50 μl isopropanol and the amounts of triglyceride and total cholesterol were determined by enzymatic colorimetric methods using the commercial kit (Asan Pharmaceutical Co.).

Histological analysis

The liver tissue was cut into 4-μm sections and stained with hematoxylin and eosin (H&E) for histopathologic analysis.

Serum and hepatic thiobarbituric acid reactive substances (TBARS) measurement

To measure hepatic TBARS, liver tissues were homogenized homogenizing buffer (10%, w:w) consisting of 154 mM KCl, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 600 × g for 10 min to obtain the supernatant. Serum and hepatic TBARS was measured according to the method of Hiroshi Ohkawa [53]. Serum or liver homogenates were mixed with 8.1% sodium dodecyl surfate (SDS), 20% acetic acid and 0.8% TBA solution. The mixture was heated at 95℃ for 60 min and then cooled immediately with ice. The red pigment was extracted with an mixture of n-butanol and pyridine (15:1, v:v) after vortexing. The mixture was centrifuged at 4000 rpm for 10 min and the supernatant was obtained. Finally, the absorbance was measured at 532 nm using 1,1,3,3-tetraethoxy -propane as a standard.

Total RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA of liver tissues was isolated using TRIzol (Invitrogen, USA) and the PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. Purified total RNA (2μg) was reverse transcribed to synthesize cDNA using Superscript II Reverse Transriptase (Invitrogen). PCR amplification mixtures (20 μl) contained 10.8 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), 0.4 μl of 10 uM reverse primer, 0.4 μl of 10 uM reverse primer, 2 μl of cDNA template, and 7.2 μl of UltraPure^{TM} Distilled Water (Invitrogen). **Table 2** depicts the primer sequences. qRT-PCR was conducted using a StepOneReal Time PCR System (Applied Biosystems) with the following cycling parameters; polymerase activation: 10 minutes, 95℃; amplification for 40 cycles: 15 seconds, 95℃; 1 minutes, 60℃. Mouse ribosomal protein L19 (RPL 19) was a used as reference gene to normalize for differences in the amount of total RNA in each sample and the relative expression levels of target genes were calculated using $2^{-\Delta\Delta Ct}$ method.

Table 2. Primer sequences for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
MCP-1	CCAGCACCAGCACCAGCCAA	TGGGGCGTTAACTGCATCTGGC
5-LO	GGCACGGCAAAAACAGTATC	TGGCATTTGGCATCAATACTC
TNFα	GGCTACAGGCTTGTCACTCGA	CACGCTCTTCTGTCTACTGAA
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
COX-2	TCTCAATGAGTACCGCAAACG	CCATTTCCTTCTCTCCTGTAAGTTC
PPARα	CCTCAGGGTACCACTACGGAGT	GCCGAATAGTTCGCCGAA
PPARγ	CCTCAGGGTACCACTACGGAGT	GCCGAATAGTTCGCCGAA
RXRα	CTTTGACAGGGTGCTAACAGAGC	ACGCTTCTAGTGACGCATACACC
LXRα	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT
FXR	TCCGGACATTCAACCATCAC	TCACTGCACATCCCAGATCTC
SREBP-1c	CTGGCACTAAGTGCCCTCAAC	GCCACATAGATCTCTGCCAGTGT
SREBP-2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
LDLR	TGGCCATCTATGAGGACAAA	GTGTGACCTTGTGGAACAGG
CD36	TCCTCTGACATTTGCAGGTCTATC	AAAGGCATTGGCTGGAAGAA
MGAT1	GCCTTGCCACTGATATATGCC	TTCAATCTGCTCTGAGGTCGG
DGAT1	GACGGCTACTGGGATCTGA	CATCACCACACACCAATTCA
SCD1	ATCTCCAGTTCTTACACGACCACC	CGTCTTCACCTTCTCTCGTTCATT
FAS	GGAGGTGGTGATAGCCGGTAT	GGGTAATCCATAGAGCCCAG
CPT1a	ACGGAGTCCTGCAACTTTGT	GTACAGGTGCTGGTGCTTTTC
HMGCR	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
ABCA1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
CYP7A1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
RPL19	TCAGGCTACAGAAGAGGCTTGC	ATCAGCCCATCCTTGATCAGC

Total protein extraction and immunoblotting

Liver tissues were homogenized in ice-cold protein lysis buffer [50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 0.1% Tween-20, 10% glycerol, 0.2 mM PMSF, protease inhibitor cocktail]. After centrifugation for 30 min at 10000 × g at 4 °C, the protein content of the supernatant was measured using protein assay kit (Bio-Rad). Equal amounts of protein were loaded into the lanes of a 12% SDS-PAGE gel, separated and then transferred to PVDF membrane. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (TTBS), the membrane was probed with a specific antibody (p-IκB; #sc-8404, Santa Cruz Biotechnology, Inc., USA and HSC 70; #sc-7298, Santa Cruz Biotechnology, Inc.) diluted in TTBS with 5% nonfat milk. Horseradish peroxidase-conjugated anti-mouse IgG was used as secondary antibody. The immunoreactive protein bands were visualized by western chemiluminescent using HRP substrate (Millipore) with exposure to X-ray film (Fuji, Japan). The bands intensities were quantified with the Quantity one software (Bio-Rad).

Statistical analysis

All data were analyzed using SPSS software (ver. 19.0, SPSS Inc.). Data were analyzed by two-way analysis of variance (ANOVA) using diet and genotype as factors. Where appropriate, one-way ANOVA followed by Duncan's multiple range test was used. Data were reported as the mean ± SEM and differences were considered significant at $P < 0.05$. Correlations between parameters were determined by Pearson′s correlation coefficient (r).

Results

Body weight and food intake

The changes of body weight are shown in **Fig. 2**. There were no statistical differences in the initial body weights of all groups (**Table 3**). During the experimental period, each group gained body weight gradually. Even though food intake was not significantly different among the groups, both WT and ApoE$^{-/-}$ mice fed an HFD supplemented with genistein had gained significantly less body weight than those fed an HFD after 5 weeks. At the end of the experiment, WT mice fed an HFD supplemented with genistein gained body weight 29.2% less than those fed an HFD and ApoE$^{-/-}$ mice fed an HFD supplemented with genistein gained body weight 18.6% less than those fed an HFD.

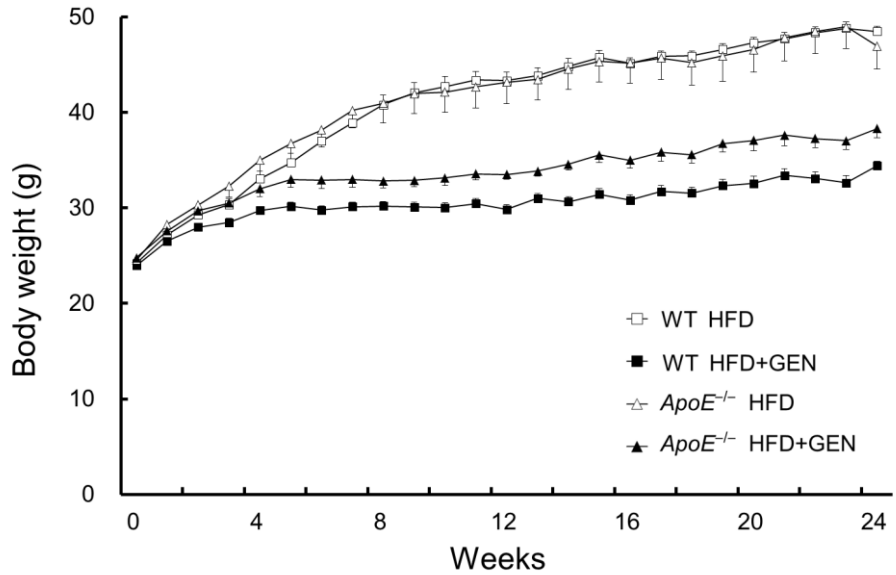


Figure 2. Changes of body weight

Body weight of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. Data are presented as means ± SEM (n = 9, 10 per group).

Table 3. Initial and final body weight, and food intake

	WT		ApoE$^{-/-}$		*P* value		
	HFD	GEN	HFD	GEN	Genotype effect	Diet effect	Interaction
Initial BW (g)	24.2 ± 0.4	24.0 ± 0.4	24.6 ± 0.7	24.8 ± 0.7	0.301	0.996	0.691
Final BW (g)	45.4 ± 0.5^{b}	32.2 ± 0.5^{a}	44.0 ± 2.3^{b}	35.8 ± 0.8^{a}	0.401	< 0.001	0.051
Food intake (g/d)	3.6 ± 0.6	3.2 ± 0.2	3.5 ± 0.2	3.4 ± 0.3	0.971	0.499	0.686

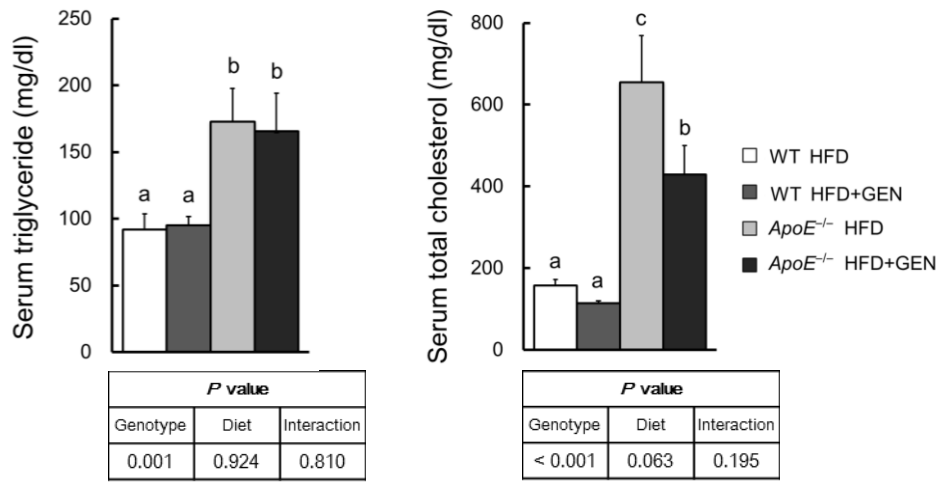
Data are presented as means ± SEM (n = 9, 10 per group). Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and interaction.

Serum biochemical analyses

Serum triglyceride and total cholesterol were significantly elevated in ApoE$^{-/-}$ mice compared with those in WT mice fed an HFD (**Fig. 3)**. Genistein supplementation significantly decreased serum total cholesterol in ApoE$^{-/-}$ mice but there were no significant differences in serum triglyceride level by genistein supplementation irrespective of genotype.

No difference in serum glucose level was observed in in ApoE$^{-/-}$ mice compared with those in WT mice fed an HFD (**Fig. 4)**. Genistein supplementation significantly reduced serum glucose in WT mice. The tendency to increase in serum insulin level and HOMA-IR was observed in ApoE$^{-/-}$ mice, which was significantly reduced by genistein.

The level of serum ALT which has been identified as a marker of liver injury was significantly higher in ApoE$^{-/-}$ mice than that in WT mice fed an HFD (**Fig. 5)**. Supplementation with genistein significantly reduced serum ALT level in ApoE$^{-/-}$ mice. Serum MCP-1 level which indicates systemic inflammation was elevated in ApoE$^{-/-}$ mice, which was significantly reduced by genistein.

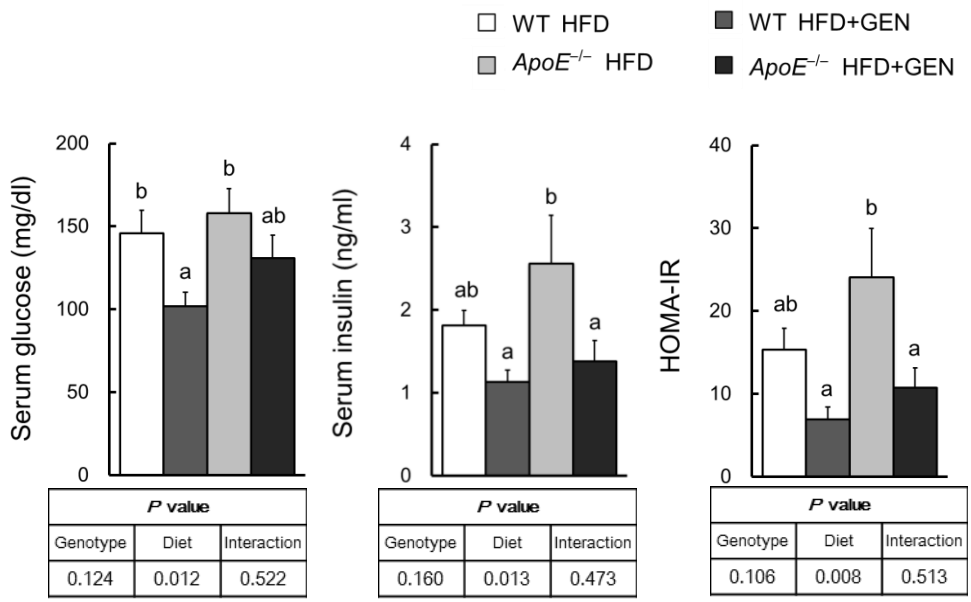


P value		
Genotype	Diet	Interaction
0.001	0.924	0.810

P value		
Genotype	Diet	Interaction
< 0.001	0.063	0.195

Figure 3. Serum lipid analyses

Serum triglyceride and total cholesterol analyses of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. Data are presented as means ± SEM (n = 5-7 per group). Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.



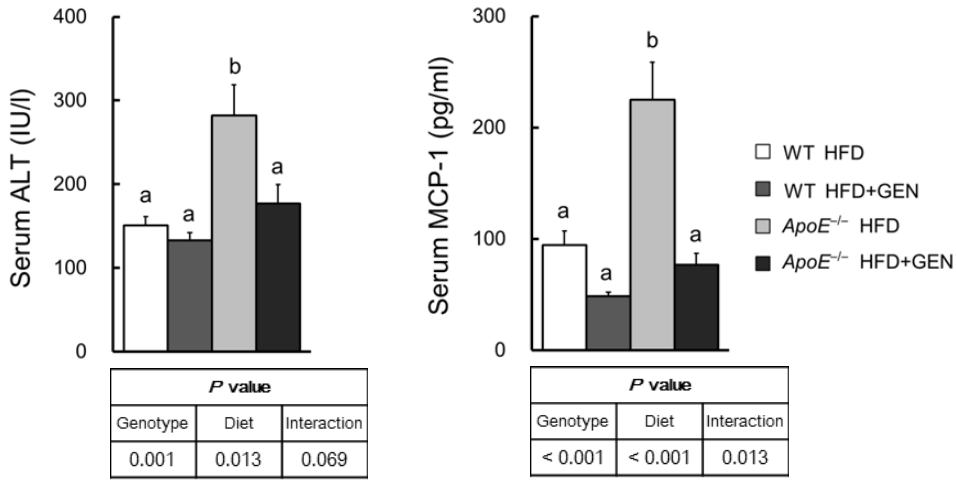
P value		
Genotype	Diet	Interaction
0.124	0.012	0.522

P value		
Genotype	Diet	Interaction
0.160	0.013	0.473

P value		
Genotype	Diet	Interaction
0.106	0.008	0.513

Figure 4. Serum glucose, insulin, and HOMA-IR analyses

Serum glucose, insulin, and HOMA-IR analyses of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. Data are presented as means ± SEM (n = 5-7 per group). Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.



P value		
Genotype	Diet	Interaction
0.001	0.013	0.069

P value		
Genotype	Diet	Interaction
< 0.001	< 0.001	0.013

Figure 5. Serum ALT and MCP-1 analyses

Serum ALT and MCP-1 analyses of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. Data are presented as means ± SEM (n = 5-7 per group). Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.

Liver weight, hepatic lipid profiles, and liver histology

Liver weight was significantly increased in ApoE$^{-/-}$ mice compared to WT mice fed an HFD (**Fig. 6)**. Genistein supplementation significantly reduced liver weight in both genotypes. Hepatic triglyceride and cholesterol levels were significantly raised in ApoE$^{-/-}$ mice compared to WT mice fed an HFD and these increases were significantly alleviated by genistein supplementation.

As shown in **Fig. 7,** H&E staining of the liver tissues depicts the parallel observation with hepatic lipid profiles. Morphological changes and extreme lipid accumulation were observed in the liver of WT and ApoE$^{-/-}$ mice fed an HFD. Furthermore, exacerbated macrovascular and microvascular steatosis as well as inflammation was observed in ApoE$^{-/-}$ mice compared to WT mice.

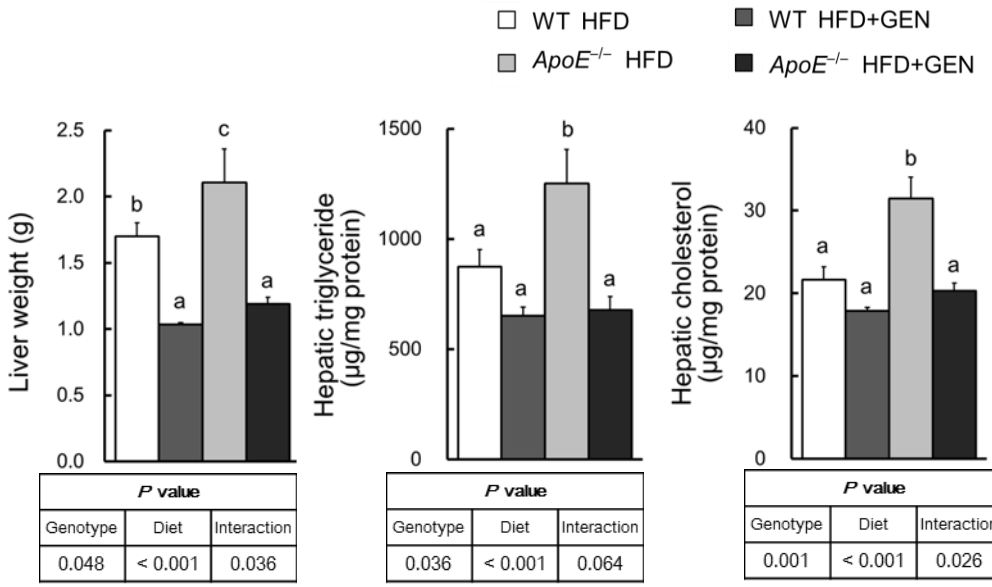


Figure 6. Liver weight, hepatic triglyceride, and heatic total cholesterol

Liver weight, hepatic triglyceride, and hepatic total cholesterol of WT and ApoE$^{-/-}$ mice fed a high-fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5g/kg diet) Data are presented as means ± SEM (n = 6, 7 per group for hepatic lipids and n = 9, 10 per group for liver weight). Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.

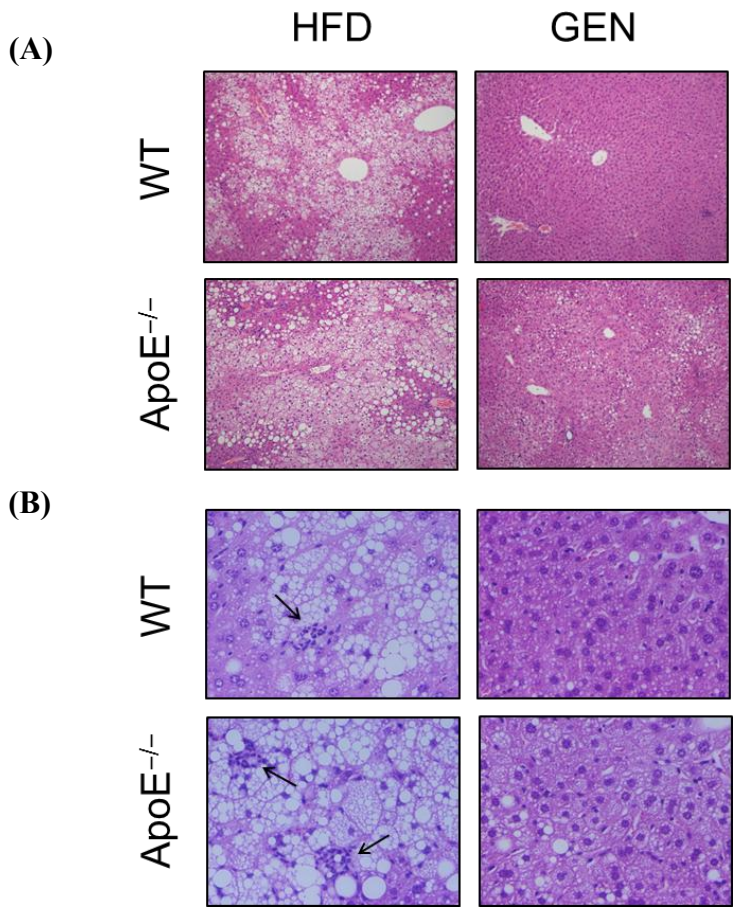


Figure 7. H&E staining of liver tissue sections

Representative H&E staining of liver tissue sections (n=3 per group, magnification 100× for A and 200× for B). Inflammation foci are indicated with an arrow

Oxidative stress and inflammation

Serum and hepatic TBARS, an indicator of lipid peroxidation, were measured to evaluate systemic and hepatic oxidative stress (**Fig. 8**). Hepatic TBARS levels showed a tendency to increase in ApoE$^{-/-}$ mice fed an HFD compared to WT mice fed an HFD. Genistein supplementation significantly decreased hepatic TBARS in ApoE$^{-/-}$ mice. Serum TBARS had a significant 27.3-fold increase in ApoE$^{-/-}$ mice compared to WT mice fed an HFD. Activation of the transcription factor nuclear factor-κB (NF-κB) in response to oxidative stress is mediated through phosphorylation of IκBs, the intracellular inhibitor for NF-κB. Hepatic p-IκBα expressions were significantly elevated in ApoE$^{-/-}$ mice compared to WT mice. Genistein significantly inhibited the NF-κB activation in ApoE$^{-/-}$ mice, as determined by the reduced expression of p-IκBα.

The hepatic mRNA expressions regarding inflammatory genes were shown in **Fig. 9**. The genes related to inflammation are MCP-1, 5-LO, TNFα, IL-1β and COX-2. MCP-1 is the key chemokine that regulates migration and infiltration of macrophages [61]. Hepatic mRNA expressions for MCP-1, 5-LO, TNFα and COX-2 were significantly up-regulated in ApoE$^{-/-}$ mice compared to WT mice fed an HFD (5.5-fold for MCP-1, 2.1-fold for 5-LO, 5.5-fold for TNFα and 5.3-fold for COX-2). The tendency

toward an increase in hepatic IL-1β expression was shown in ApoE$^{-/-}$ mice compared to WT mice (1.8-fold for IL-1β). Hepatic MCP-1, TNFα and COX-2 expression levels significantly decreased in ApoE$^{-/-}$ mice by genistein supplementation.

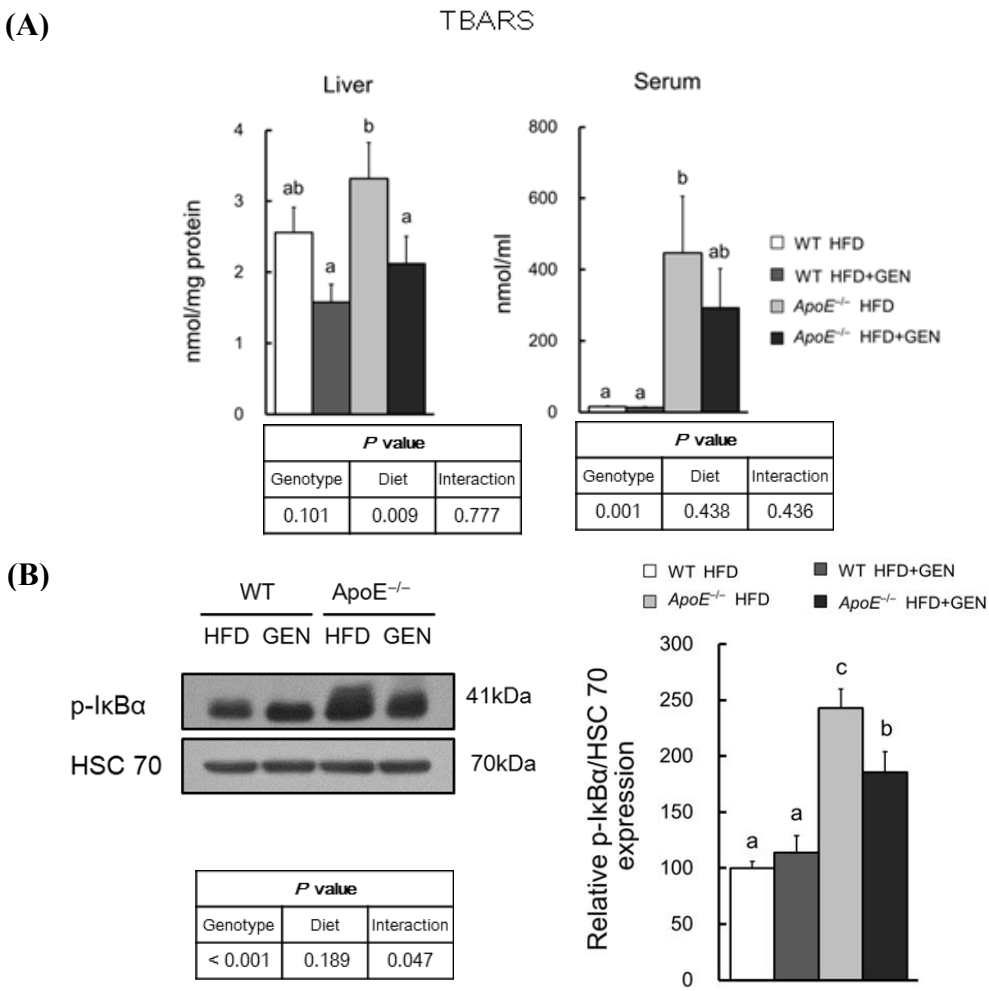
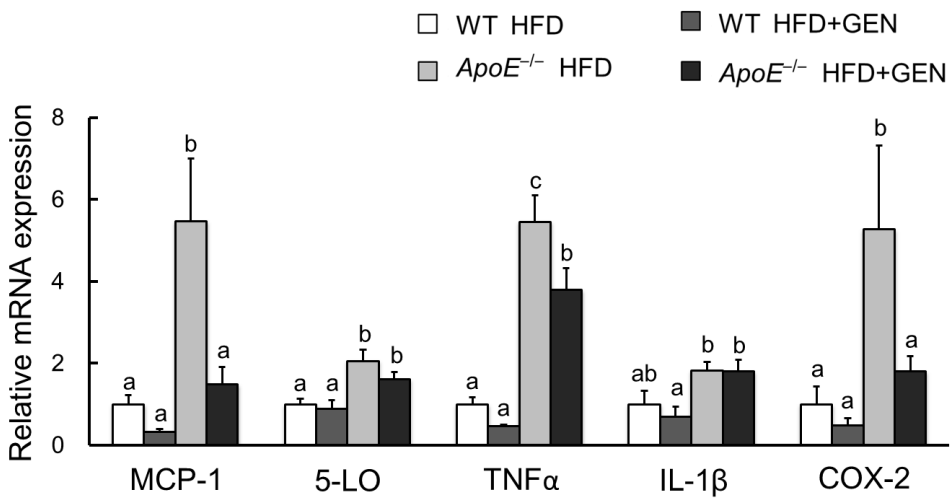


Figure 8. Serum and hepatic lipid peroxidation and hepatic NF-κB activation
(A) Serum and hepatic TBARS (n = 5-7 per group). **(B)** Western blotting for p-IκBα with its densitometric analysis (n = 3 per group). Relative protein expression levels of p-IκBα/HSC 70 were quantitated. Data are presented as means ± SEM. Bars with different superscripts are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.



P value		MCP-1	5-LO	TNFa	IL-1b	COX-2
	Genotype	0.002	< 0.001	< 0.001	0.002	0.013
	Diet	0.007	0.188	0.013	0.548	0.065
	Interaction	0.044	0.415	0.168	0.611	0.162

Figure 9. Relative expression of hepatic genes involved in inflammation

The expression was determined with real-time PCR and was normalized to RPL19 mRNA expression, where the values for WT mice fed an HFD were set 1.0. Data are expressed as means ± SEM (n = 5, 6). Bars with different superscripts within the same gene are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.

Correlation between serum total cholesterol and serum and hepatic inflammatory parameters

Serum ALT and serum MCP-1 levels were strongly correlated with serum total cholesterol as shown in **Fig. 10**. In addition, serum total cholesterol levels were strongly associated with NF-κB activation and proinflammatory gene expressions in the liver (**Fig. 11**). These findings suggest that serum total cholesterol may be the strong risk factor in accelerating NASH progression.

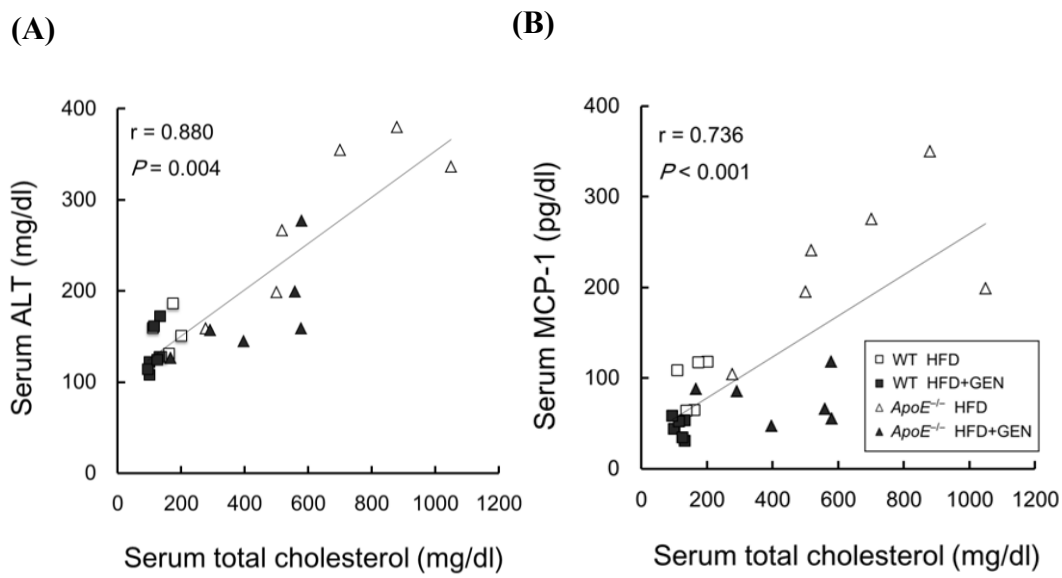


Figure 10. Relation between serum parameters and serum total cholesterol

Pearson's correlation between **(A)** serum ALT and serum cholesterol, and **(B)** serum MCP-1 and serum cholesterol. Pearson correlation coefficient, r, and *P*-value are indicated for each region.

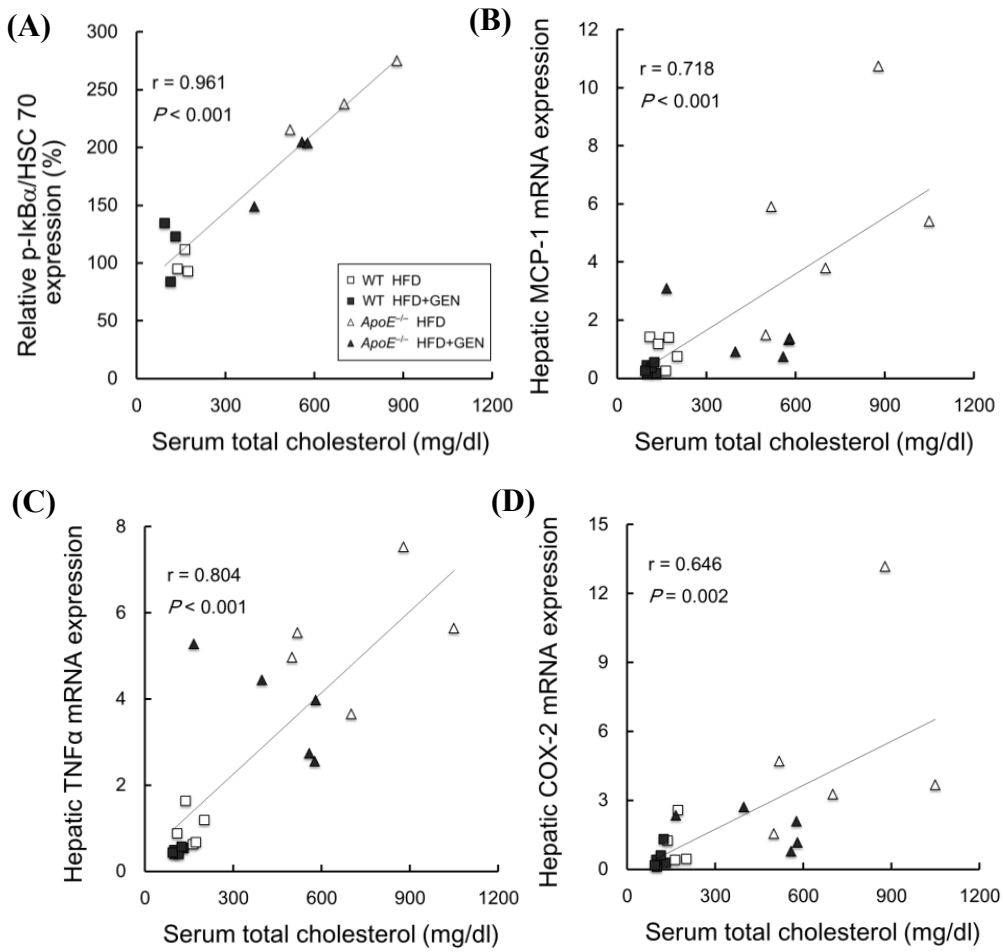


Figure 11. Relation between hepatic inflammation and serum total cholesterol

Relation between **(A)** serum total cholesterol levels and relative p-IκBα protein levels, **(B)** serum total cholesterol levels and hepatic monocyte chemoattractant protein 1 (MCP-1) mRNA levels, **(C)** serum total cholesterol levels and hepatic tumor necrosis factor alpha (TNFα) mRNA levels, and **(D)** serum total cholesterol levels and hepatic cyclooxygenase 2 (COX-2) mRNA levels. Pearson correlation coefficient, *r,* and P-value are indicated for each region (A: n = 3 per group, B-D: n = 5, 6 per group).

Expressions of genes involved in lipid metabolism in the liver

To investigate the effect of genistein on lipid metabolism in the liver, mRNA levels of transcription factors were determined. Hepatic mRNA expressions for transcriptions factors for including PPARα, PPARγ, RXRα, LXRα, FXR, SREBP-1c and SREBP-2 are shown in **Fig. 12**. There were no significant differences in hepatic mRNA expressions of PPARα, RXRα, FXR and SREBP-1c among the groups. Compared to WT mice, hepatic mRNA levels of PPARα and SREBP-2 were lower in magnitude in ApoE$^{-/-}$ mice (genotype effect P = 0.024 and P = 0.012, respectively). Among the transcription factors, only PPARγ expressions were significantly affected by genistein supplementation (diet effect P = 0.003).

Hepatic mRNA expressions for lipid receptors including LDLR and CD36 are shown in **Fig. 13.** Hepatic LDLR mRNA expression in ApoE$^{-/-}$ mice decreased compared to diet-matched WT mice (genotype effect *P* =0.021), however, there was an induction of CD36 mRNA abundance in ApoE$^{-/-}$ mice (genotype effect P = 0.022). CD36 which is a scavenger receptor mediating uptake of oxLDL is controlled by PPARγ. Genistein supplementation reduced CD36 mRNA expression in both WT and ApoE$^{-/-}$ mice (diet effect *P* =0.022).

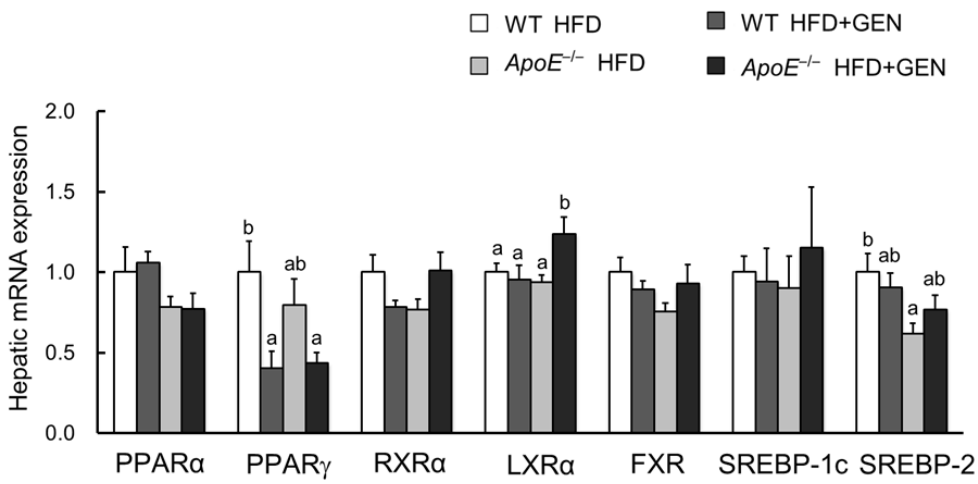
Hepatic mRNA expressions for enzymes involved in triglyceride

synthesis are shown in **Fig. 14.** Hepatic monoacylglycerol O-acyltransferase 1 (MGAT1) was identified to be regulated by PPARγ [54] and its expression was significantly decreased in both WT and ApoE$^{-/-}$ mice in response to genistein. There were no significant differences in hepatic mRNA expressions of diacylglycerol acyltransferase 1 (DGAT1). Hepatic stearoyl-CoA desaturase-1 (SCD1) expression was significantly decreased in WT mice compared to ApoE$^{-/-}$ mice, suggesting that hepatic de novo lipogenesis would be a primary pathway for accumulating hepatic triglyceride contents in WT mice. Hepatic SCD1 expressions significantly decreased only in WT mice by genistein. We failed to detect differences in fatty acid oxidation and fatty acid synthesis among the groups as determined by carnitine palmitoyltransferase 1a (CPT1a) mRNA levels and fatty acid synthase (FAS).

The correlations between hepatic mRNA levels involved in PPARγ and its target genes and hepatic triglyceride content are shown in **Fig. 15**. Hepatic PPARγ mRNA level was correlated with hepatic triglyceride content. Hepatic CD36 mRNA and hepatic MGAT1 mRNA levels were significantly correlated with hepatic triglyceride content. These findings suggest that hepatic PPARγ and its target genes would contribute to the TG accumulation in the liver.

The hepatic relative expressions of genes involved in cholesterol

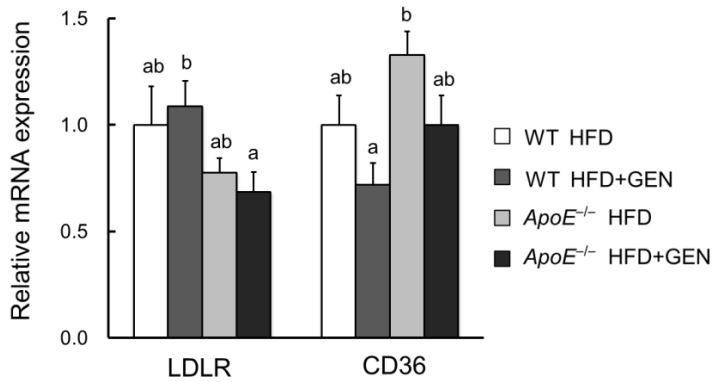
metabolism are shown in **Fig. 16.** Genistein tended to increase cholesterol 7α-hydroxylase (CYP7A1) mRNA expressions in ApoE$^{-/-}$ mice fed an HFD. A strong negative correlation between hepatic CYP7A1 mRNA levels and hepatic cholesterol was observed ($r = -0.665$, $P = 0.036$), suggesting that elevated bile acid synthesis may be involved in the hypocholesterolemic effect of genistein in ApoE$^{-/-}$ mice. There were no differences in hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and adenosine triphosphate-binding cassette A1 (ABCA1) mRNA expression among the groups.



P value		PPARα	PPARγ	RXRα	LXRα	FXR	SREBP-1c	SREBP-2
	Genotype	0.024	0.544	0.959	0.125	0.223	0.822	0.012
	Diet	0.817	0.003	0.869	0.179	0.693	0.694	0.774
	Interaction	0.747	0.405	0.015	0.041	0.102	0.538	0.201

Figure 12. Relative expression of hepatic genes involved in transcription factors

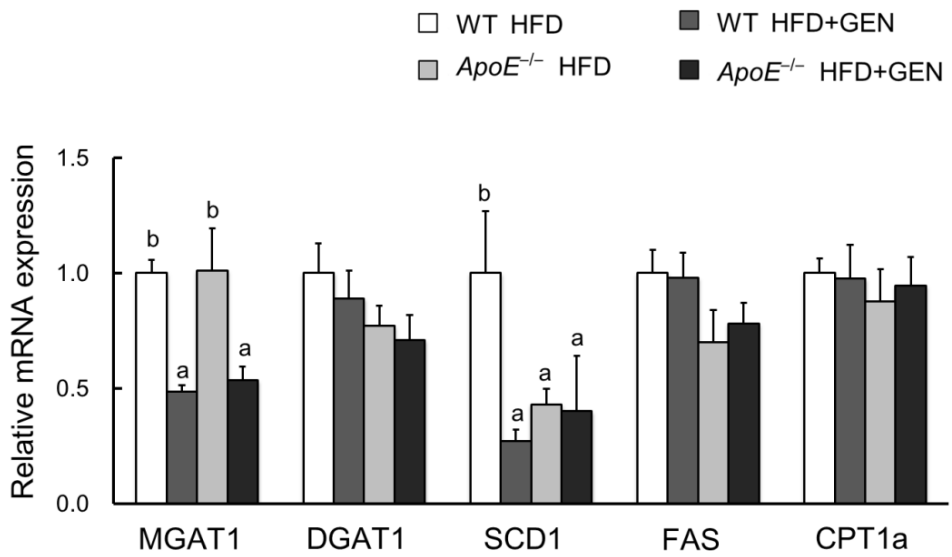
Hepatic mRNA expression of genes encoding transcription factors of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. The expression was determined with real-time PCR and was normalized to RPL19 mRNA expression, where the values for WT mice fed an HFD were set 1.0. Data are expressed as means ± SEM (n = 5, 6). Bars with different superscripts within the same gene are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.



P value		LDLR	CD36
	Diet	0.983	0.022
	Genotype	0.021	0.022
	Interaction	0.478	0.824

Figure 13. Relative expression of hepatic genes involved in lipoprotein receptors

Hepatic mRNA expression of genes encoding lipid receptors of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. The expression was determined with real-time PCR and was normalized to RPL19 mRNA expression, where the values for WT mice fed an HFD were set 1.0. Data are expressed as means ± SEM (n = 5, 6). Bars with different superscripts within the same gene are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.



P value	MGAT1	DGAT1	SCD1	FAS	CPT1a
Genotype	0.766	0.093	0.226	0.042	0.557
Diet	<0.001	0.472	0.045	0.790	0.870
Interaction	0.855	0.797	0.068	0.689	0.723

Figure 14. Relative expression of hepatic genes involved in triglyceride metabolism

Hepatic mRNA expression of genes involved in triglyceride metabolism of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and an HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. The expression was determined with real-time PCR and was normalized to RPL19 mRNA expression, where the values for WT mice fed an HFD were set 1.0. Data are expressed as means ± SEM (n = 5, 6). Bars with different superscripts within the same gene are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.

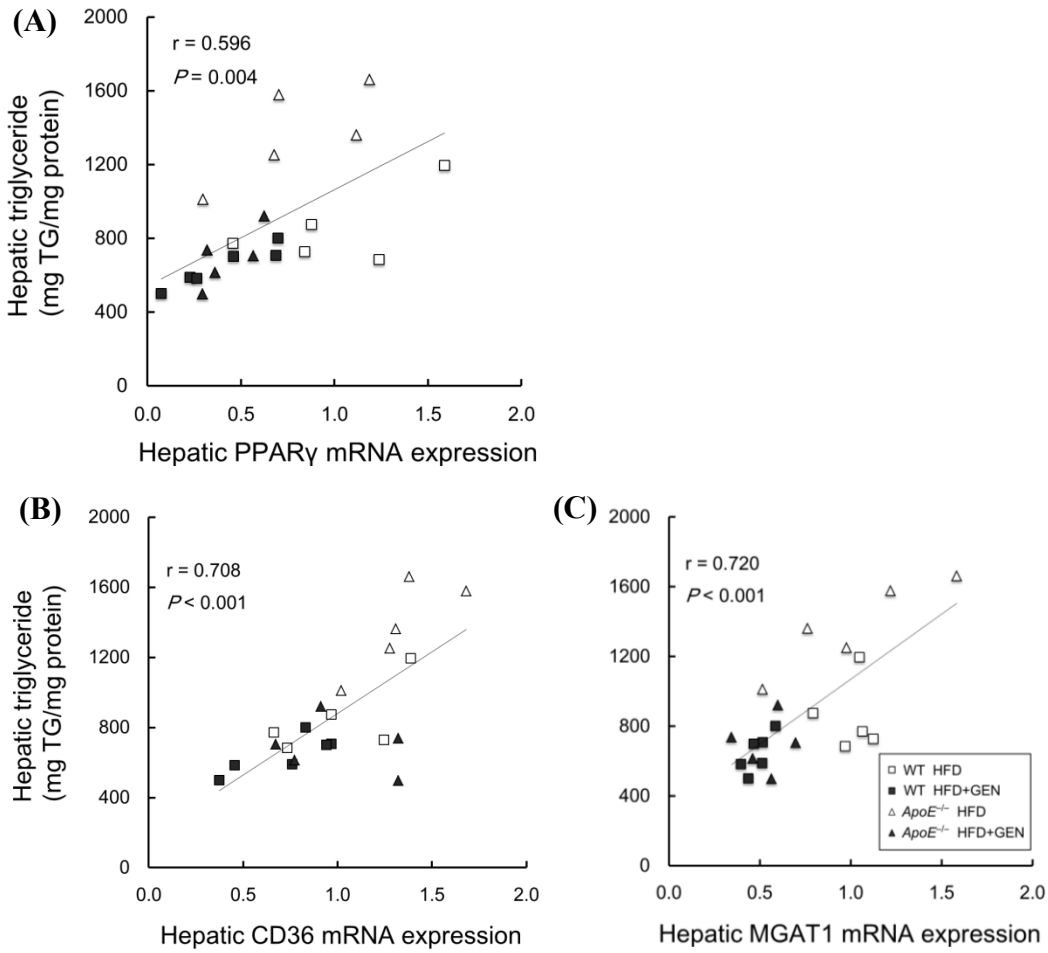
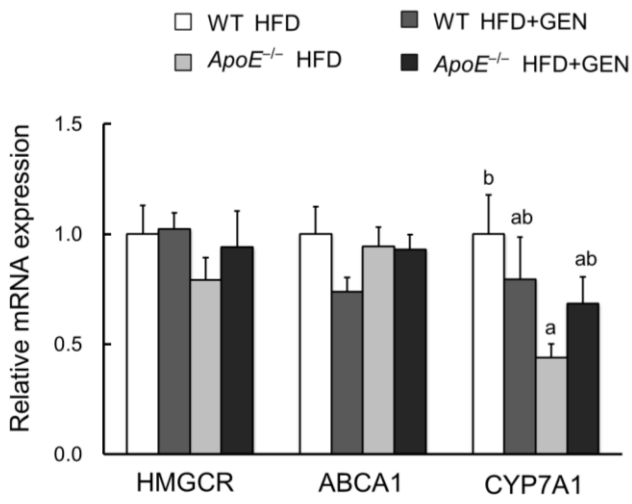


Figure 15. Relation between PPARγ and its target genes expressions and hepatic triglyceride levels

Relation **(A)** between hepatic triglyceride levels and hepatic PPARγ, **(B)** between hepatic triglyceride levels and hepatic CD36, and **(C)** between hepatic triglyceride levels and MGAT1 mRNA levels. Pearson correlation coefficient, r, and P-value are indicated for each region (n = 5, 6 per group)



P value		HMGCR	ABCA1	CYP7A1
	Genotype	0.233	0.450	0.308
	Diet	0.479	0.133	0.161
	Interaction	0.599	0.177	0.090

Figure 16. Relative expression of hepatic genes involved in cholesterol metabolism

Hepatic mRNA expression of genes involved in triglyceride metabolism of WT and ApoE$^{-/-}$ mice fed a high fat diet (HFD) and HFD supplemented with genistein (GEN) (0.5 g per kg diet) for 24 weeks. The expression was determined with real-time PCR and was normalized to RPL19 mRNA expression, where the values for WT mice fed an HFD were set 1.0. Data are expressed as means ± SEM (n = 5, 6). Bars with different superscripts within the same gene are significantly different at $P < 0.05$. All measurements were compared by two-way ANOVA for main effect (diet and genotype) and an interaction.

Discussion

Dietary genistein has shown several biological actions with regard to inhibiting NAFLD in high-fat induced obese rats and mice [55-57]. However, there is little study regarding the effect of genistein on a NASH model using hyperlipidemic ApoE$^{-/-}$ mice. Thus we aimed to investigate the effect of genistein in ApoE$^{-/-}$ mice fed an HFD for 24 weeks. In the present study, we observed that genistein alleviated NASH development by restoring impaired cholesterol metabolism and by reducing oxidized lipoprotein-mediated triglyceride accumulation and inflammation. In addition, we confirmed that ApoE$^{-/-}$ mice were more sensitized to HFD-induced NASH development than WT mice.

Previous studies show that serum levels of TBARS and oxLDL, different measures of lipid peroxidation are highly correlated. In NASH patients, a strong correlation between serum TBARS and oxLDL were observed and these levels of lipid peroxidation reflected the level of NASH [58]. Moreoever, in the state of hyperlipidemia on ApoE$^{-/-}$ mice fed a normal-fat diet, the plasma of oxLDL was gradually increased from 4 weeks to 20 weeks [59]. Accordingly, we observed significant elevation in hepatic lipid peroxidation and proinflammartory gene expressions in ApoE$^{-/-}$ mice. When mice were fed an HFD, ApoE$^{-/-}$ mice exhibited increased NF-

κB activation and following higher hepatic levels of inflammatory genes, suggesting that inflammation plays a pivotal role in the pathogenesis of NASH development. MCP-1 has been suggested as indicator of NASH. In NASH patients, serum MCP-1 level was aligned with the level of NASH [53]. MCP-1 is a potent chemoattractant protein to maintain the inflammatory infiltrate during liver injury. In this study, genistein reduced both heapatic MCP-1 mRNA level as well as serum MCP-1. In previous study, genistein-treated THP-1 cells showed inhibition of MCP-1 mRNA expression [54], suggesting that genistein can play a role in inhibiting MCP-1 expression. The result of serum and hepatic TBARS in this study indicated that genistein supplementation plays an important role as antioxidant and decreased oxidative stress. It is reported that soy isoflavones attenuated oxidative stress in livers of obese rats [69]. However, the levels of other oxidation products including 4-HNE and protein carbonyl groups in liver were not affected by genistein.

OxLDL is recognized as high affinity PPARγ ligands and agonists [60] and CD36 is one of the scavenger receptors mediating uptake of oxLDL and a target gene for PPARγ [61]. We found that hepatic PPARγ expression and CD36 expression were decreased by genistein supplementation, which might lead to inhibit the uptake of oxLDL into KCs. Thus, we demonstrate that genistein may improve oxLDL-PPARγ-CD36

feed-forward cycle. Recent study has been demonstrated that when oxLDL is internalized, it is transported to the lysosomal compartment where it is poorly degraded or hydrolyzed and therefore accumulates in lysosomes [62]. Decreased PPARγ and CD36 expressions in response to genistein suggest that genistein may decrease hepatic lipid accumulation by reducing oxLDL uptake. In support of this view, the mRNA levels of MGAT1, another target gene for PPARγ, were also increased in the livers of ApoE$^{-/-}$ mice and hepatic MGAT1 expressions were decreased by genistein supplementation. Recent study has shown that knockdown of hepatic MGAT1 declined hepatic steatosis, suggesting that the MGAT1 induced by hepatic PPARγ plays an important role in the development of hepatic steatosis [32]. In contrast, genes regarding de novo lipogenesis (SREBP-1c, SCD1, FAS and DGAT1) and fatty acid oxidation (PPARα, CPT1a) were not changed by genistein in ApoE$^{-/-}$ mice. The previous study reported that when mice were fed an HFD (36% of total energy) supplemented with 2% genistein for 12 weeks, hepatic expressions of PPARα and its target genes, CPTs were increased [63].

We also found that hypocholesterolemic effect and anti-inflammatory effect by genistein played an crucial role in the inhibition of NASH development in ApoE$^{-/-}$ mice. This effect can be explained by increased hepatic CYP7A1 expression in apoE$^{-/-}$ mice contributing to increase bile

acid synthesis and remove cholesterol into bile, resulting in reduced hepatic cholesterol accumulation. Genistein did not alter cholesterol uptake, biosynthesis, or efflux, as determined by mRNA expression of genes, including LDLR, HMGCR, and ABCA1. We observed that the serum total cholesterol levels are highly correlated with hepatic inflammatory genes. Similarly, previous study demonstrated that cholesterol-derived products (i.e., oxysterols) are able to induce proinflammatory mechanism in liver cells [11]. Moreover, it has been reported that highly oxidized LDL particle turns into a structure similar to pathogen-related epitopes and therefore will be removed from plasma through uptake by macrophage. When excessive lipid is accumulated, the macrophage are changed into foam cell [64] and this change can induce the production of inflammatory cytokines [65]. This observation indicates that hypocholesterolemic genistein contributes to inhibit foam cell formation via inhibiting uptake of oxLDL and therefore decreases the production of inflammatory markers.

A previous study reported that mice fed AIN-93G diet supplemented with various dosages of genistein (125–1000 g/kg diet) for 24 weeks exhibited 0.39–3.36 μmol/L levels of plasma total genistein, which are similar with those detected in women consuming soy products [66], implicating that the plasma genistein in response to 500g genistein/kg diet used in this study would be within the physiological concentration range.

In conclusion, it is clear that dietary supplementation of genistein favorably affected cholesterol homeostasis and inhibited the up-regulation of hepatic proinflammatory genes in ApoE$^{-/-}$ mice fed an HFD. Given that oxLDL has been shown to exert potent inflammatory effects, elevated lipid peroxidation due to the increased serum cholesterol levels and oxidative stress may trigger hepatic inflammation in ApoE$^{-/-}$ mice by NF-κB activation. Therefore, our findings provide novel insights into the effects of genistein restoring altered cholesterol metabolism and oxidative stress against NASH development.

References

1. Neuschwander-Tetri BA, Caldwell SH: **Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference.** *Hepatology* 2003, **37:**1202-1219.
2. Byrne CD, Olufadi R, Bruce KD, Cagampang FR, Ahmed MH: **Metabolic disturbances in non-alcoholic fatty liver disease.** *Clinical Science* 2009, **116:**539-564.
3. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A: **The natural history of nonalcoholic fatty liver disease: a population-based cohort study.** *Gastroenterology* 2005, **129:**113-121.
4. Chitturi S, Farrell GC, George J: **Non-alcoholic steatohepatitis in the Asia-Pacific region: future shock?** *J Gastroenterol Hepatol* 2004, **19:**368-374.
5. Park SH, Jeon WK, Kim SH, Kim HJ, Park DI, Cho YK, Sung IK, Sohn CI, Keum DK, Kim BI: **Prevalence and risk factors of non-alcoholic fatty liver disease among Korean adults.** *J Gastroenterol Hepatol* 2006, **21:**138-143.
6. Tsochatzis EA, Papatheodoridis GV: **Is there any progress in the treatment of non-alcoholic fatty liver disease?** *World J Gastrointest Pharmacol Ther* 2011, **2:**1-5.
7. Day CP, James OF: **Steatohepatitis: a tale of two "hits"?** *Gastroenterology* 1998, **114:**842-845.
8. Parekh S, Anania FA: **Abnormal lipid and glucose metabolism in obesity: implications for nonalcoholic fatty liver disease.** *Gastroenterology* 2007, **132:**2191-2207.
9. Neuschwander-Tetri BA: **Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites.** *Hepatology* 2010, **52:**774-788.
10. Mari M, Caballero F, Colell A, Morales A, Caballeria J, Fernandez A, Enrich C, Fernandez-Checa JC, Garcia-Ruiz C: **Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis.** *Cell Metab* 2006, **4:**185-198.
11. Ferre N, Martinez-Clemente M, Lopez-Parra M, Gonzalez-Periz A, Horrillo R, Planaguma A, Camps J, Joven J, Tres A, Guardiola F, et al: **Increased susceptibility to exacerbated liver injury in hypercholesterolemic ApoE-deficient mice: potential involvement of**

oxysterols. *Am J Physiol Gastrointest Liver Physiol* 2009, **296:**G553-562.

12. Bieghs V, Wouters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, Lutjohann D, Febbraio M, Moore KJ, van Bilsen M, et al: **Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice.** *Gastroenterology* 2010, **138:**2477-2486, 2486 e2471-2473.
13. Martinez-Clemente M, Ferre N, Gonzalez-Periz A, Lopez-Parra M, Horrillo R, Titos E, Moran-Salvador E, Miquel R, Arroyo V, Funk CD, Claria J: **5-Lipoxygenase deficiency reduces hepatic inflammation and tumor necrosis factor alpha-induced hepatocyte damage in hyperlipidemia-prone ApoE-null mice.** *Hepatology* 2010, **51:**817-827.
14. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, Kerksiek A, van Kruchten R, Maeda N, Staels B, et al: **Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis.** *Hepatology* 2008, **48:**474-486.
15. Tous M, Ferre N, Rull A, Marsillach J, Coll B, Alonso-Villaverde C, Camps J, Joven J: **Dietary cholesterol and differential monocyte chemoattractant protein-1 gene expression in aorta and liver of apo E-deficient mice.** *Biochem Biophys Res Commun* 2006, **340:**1078-1084.
16. Hodis HN, Kramsch DM, Avogaro P, Bittolo-Bon G, Cazzolato G, Hwang J, Peterson H, Sevanian A: **Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL-).** *J Lipid Res* 1994, **35:**669-677.
17. Holvoet P, Theilmeier G, Shivalkar B, Flameng W, Collen D: **LDL Hypercholesterolemia Is Associated With Accumulation of Oxidized LDL, Atherosclerotic Plaque Growth, and Compensatory Vessel Enlargement in Coronary Arteries of Miniature Pigs.** *Arterioscler Thromb Vasc Biol* 1998, **18:**415-422.
18. van Tits LJ, van Himbergen TM, Lemmers HL, de Graaf J, Stalenhoef AF: **Proportion of oxidized LDL relative to plasma apolipoprotein B does not change during statin therapy in patients with heterozygous familial hypercholesterolemia.** *Atherosclerosis* 2006, **185:**307-312.
19. Li D, Mehta JL: **Oxidized LDL, a critical factor in atherogenesis.** *Cardiovasc Res* 2005, **68:**353-354.
20. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, Muls E, Van de Werf F: **Circulating Oxidized LDL Is a Useful Marker for Identifying Patients With Coronary Artery Disease.** *Arterioscler Thromb Vasc Biol* 2001, **21:**844-848.

21. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM: **Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma.** *Cell* 1998, **93:**229-240.
22. Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE: **Regulation of PPAR gamma gene expression by nutrition and obesity in rodents.** *J Clin Invest* 1996, **97:**2553-2561.
23. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer B, Jr., Reitman ML, Gonzalez FJ: **Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes.** *J Clin Invest* 2003, **111:**737-747.
24. Schadinger SE, Bucher NL, Schreiber BM, Farmer SR: **PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes.** *Am J Physiol Endocrinol Metab* 2005, **288:**E1195-1205.
25. Matsusue K, Kusakabe T, Noguchi T, Takiguchi S, Suzuki T, Yamano S, Gonzalez FJ: **Hepatic steatosis in leptin-deficient mice is promoted by the PPARgamma target gene Fsp27.** *Cell Metab* 2008, **7:**302-311.
26. Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, Londos C: **Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis.** *J Biol Chem* 2000, **275:**38486-38493.
27. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW: **Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages.** *J Biol Chem* 2002, **277:**49982-49988.
28. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A, Pastorino AM, Lo Cascio V, Sawamura T: **Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species.** *J Biol Chem* 2000, **275:**12633-12638.
29. Tang FT, Cao Y, Wang TQ, Wang LJ, Guo J, Zhou XS, Xu SW, Liu WH, Liu PQ, Huang HQ: **Tanshinone IIA attenuates atherosclerosis in ApoE(-/-) mice through down-regulation of scavenger receptor expression.** *Eur J Pharmacol* 2011, **650:**275-284.
30. Navab M, Imes SS, Hama SY, Hough GP, Ross LA, Bork RW, Valente AJ, Berliner JA, Drinkwater DC, Laks H, et al.: **Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic**

protein 1 synthesis and is abolished by high density lipoprotein. *J Clin Invest* 1991, **88:**2039-2046.

31. Clarke MC, Talib S, Figg NL, Bennett MR: **Vascular smooth muscle cell apoptosis induces interleukin-1-directed inflammation: effects of hyperlipidemia-mediated inhibition of phagocytosis.** *Circ Res* 2010, **106:**363-372.
32. Lee YJ, Ko EH, Kim JE, Kim E, Lee H, Choi H, Yu JH, Kim HJ, Seong J-K, Kim K-S, Kim J-w: **Nuclear receptor PPARγ-regulated monoacylglycerol O-acyltransferase 1 (MGAT1) expression is responsible for the lipid accumulation in diet-induced hepatic steatosis.** *Proceedings of the National Academy of Sciences* 2012, **109:**13656-13661.
33. Lohmann C, Schafer N, von Lukowicz T, Sokrates Stein MA, Boren J, Rutti S, Wahli W, Donath MY, Luscher TF, Matter CM: **Atherosclerotic mice exhibit systemic inflammation in periadventitial and visceral adipose tissue, liver, and pancreatic islets.** *Atherosclerosis* 2009, **207:**360-367.
34. Sinha-Hikim I, Sinha-Hikim AP, Shen R, Kim H, French SW, Vaziri ND, Crum A, Rajavashisth TB, Norris KC: **A novel cystine based antioxidant attenuates oxidative stress and hepatic steatosis in diet-induced obese mice.** *Exp Mol Pathol* 2011, **91:**419-428.
35. Daugherty A: **Mouse models of atherosclerosis.** *Am J Med Sci* 2002, **323:**3-10.
36. Mensenkamp AR, Havekes LM, Romijn JA, Kuipers F: **Hepatic steatosis and very low density lipoprotein secretion: the involvement of apolipoprotein E.** *J Hepatol* 2001, **35:**816-822.
37. Hashimoto E, Tokushige K: **Prevalence, gender, ethnic variations, and prognosis of NASH.** *J Gastroenterol* 2011, **46 Suppl 1:**63-69.
38. Shwaery GT, Vita JA, Keaney JF, Jr.: **Antioxidant protection of LDL by physiological concentrations of 17 beta-estradiol. Requirement for estradiol modification.** *Circulation* 1997, **95:**1378-1385.
39. Uzui H, Sinha SK, Rajavashisth TB: **17beta-estradiol inhibits oxidized low-density lipoprotein-induced increase in matrix metalloproteinase-9 expression in human macrophages.** *J Investig Med* 2011, **59:**1104-1108.
40. Sack MN, Rader DJ, Cannon RO, 3rd: **Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women.** *Lancet* 1994, **343:**269-270.
41. Reinli K, Block G: **Phytoestrogen content of foods--a compendium of literature values.** *Nutr Cancer* 1996, **26:**123-148.
42. Crisafulli A, Altavilla D, Marini H, Bitto A, Cucinotta D, Frisina N,

Corrado F, D'Anna R, Squadrito G, Adamo EB, et al: **Effects of the phytoestrogen genistein on cardiovascular risk factors in postmenopausal women.** *Menopause* 2005, **12:**186-192.

43. Naaz A, Yellayi S, Zakroczymski MA, Bunick D, Doerge DR, Lubahn DB, Helferich WG, Cooke PS: **The soy isoflavone genistein decreases adipose deposition in mice.** *Endocrinology* 2003, **144:**3315-3320.
44. Wei H, Bowen R, Cai Q, Barnes S, Wang Y: **Antioxidant and antipromotional effects of the soybean isoflavone genistein.** *Proc Soc Exp Biol Med* 1995, **208:**124-130.
45. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y: **Genistein, a specific inhibitor of tyrosine-specific protein kinases.** *J Biol Chem* 1987, **262:**5592-5595.
46. Gong L, Li Y, Nedeljkovic-Kurepa A, Sarkar FH: **Inactivation of NF-kappaB by genistein is mediated via Akt signaling pathway in breast cancer cells.** *Oncogene* 2003, **22:**4702-4709.
47. Horia E, Watkins BA: **Complementary actions of docosahexaenoic acid and genistein on COX-2, PGE2 and invasiveness in MDA-MB-231 breast cancer cells.** *Carcinogenesis* 2006, **28:**809-815.
48. De Paula ML, Rodrigues DH, Teixeira HC, Barsante MM, Souza MA, Ferreira AP: **Genistein down-modulates pro-inflammatory cytokines and reverses clinical signs of experimental autoimmune encephalomyelitis.** *Int Immunopharmacol* 2008, **8:**1291-1297.
49. Yalniz M, Bahcecioglu IH, Kuzu N, Poyrazoglu OK, Bulmus O, Celebi S, Ustundag B, Ozercan IH, Sahin K: **Preventive role of genistein in an experimental non-alcoholic steatohepatitis model.** *J Gastroenterol Hepatol* 2007, **22:**2009-2014.
50. Ji G, Yang Q, Hao J, Guo L, Chen X, Hu J, Leng L, Jiang Z: **Anti-inflammatory effect of genistein on non-alcoholic steatohepatitis rats induced by high fat diet and its potential mechanisms.** *Int Immunopharmacol* 2011, **11:**762-768.
51. Takahashi Y, Odbayar TO, Ide T: **A comparative analysis of genistein and daidzein in affecting lipid metabolism in rat liver.** *J Clin Biochem Nutr* 2009, **44:**223-230.
52. Folch J, Lees M, Stanley GHS: **A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES.** *Journal of Biological Chemistry* 1957, **226:**497-509.
53. Ohkawa H, Ohishi N, Yagi K: **Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction.** *Anal Biochem* 1979, **95:**351-358.
54. Lee YJ, Ko EH, Kim JE, Kim E, Lee H, Choi H, Yu JH, Kim HJ, Seong

JK, Kim KS, Kim JW: **Nuclear receptor PPARgamma-regulated monoacylglycerol O-acyltransferase 1 (MGAT1) expression is responsible for the lipid accumulation in diet-induced hepatic steatosis.** *Proc Natl Acad Sci U S A* 2012, **109:**13656-13661.

55. Lee YM, Choi JS, Kim MH, Jung MH, Lee YS, Song J: **Effects of dietary genistein on hepatic lipid metabolism and mitochondrial function in mice fed high-fat diets.** *Nutrition* 2006, **22:**956-964.
56. Kim MH, Kang KS, Lee YS: **The inhibitory effect of genistein on hepatic steatosis is linked to visceral adipocyte metabolism in mice with diet-induced non-alcoholic fatty liver disease.** *Br J Nutr* 2010, **104:**1333-1342.
57. Ji G, Yang Q, Hao J, Guo L, Chen X, Hu J, Leng L, Jiang Z: **Anti-inflammatory effect of genistein on non-alcoholic steatohepatitis rats induced by high fat diet and its potential mechanisms.** *International Immunopharmacology* 2011, **11:**762-768.
58. Chalasani N, Deeg MA, Crabb DW: **Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis.** *Am J Gastroenterol* 2004, **99:**1497-1502.
59. Kato R, Mori C, Kitazato K, Arata S, Obama T, Mori M, Takahashi K, Aiuchi T, Takano T, Itabe H: **Transient increase in plasma oxidized LDL during the progression of atherosclerosis in apolipoprotein E knockout mice.** *Arterioscler Thromb Vasc Biol* 2009, **29:**33-39.
60. Davies SS, Pontsler AV, Marathe GK, Harrison KA, Murphy RC, Hinshaw JC, Prestwich GD, Hilaire AS, Prescott SM, Zimmerman GA, McIntyre TM: **Oxidized alkyl phospholipids are specific, high affinity peroxisome proliferator-activated receptor gamma ligands and agonists.** *J Biol Chem* 2001, **276:**16015-16023.
61. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM: **PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation.** *Nat Med* 2001, **7:**48-52.
62. Jerome WG, Cox BE, Griffin EE, Ullery JC: **Lysosomal cholesterol accumulation inhibits subsequent hydrolysis of lipoprotein cholesteryl ester.** *Microsc Microanal* 2008, **14:**138-149.
63. Kim S, Sohn I, Lee YS: **Hepatic gene expression profiles are altered by genistein supplementation in mice with diet-induced obesity.** *J Nutr* 2005, **135:**33-41.
64. Itabe H, Obama T, Kato R: **The Dynamics of Oxidized LDL during Atherogenesis.** *J Lipids* 2011, **2011:**418313.

65. Groeneweg M, Kanters E, Vergouwe MN, Duerink H, Kraal G, Hofker MH, de Winther MPJ: **Lipopolysaccharide-induced gene expression in murine macrophages is enhanced by prior exposure to oxLDL.** *J Lipid Res* 2006, **47:**2259-2267.

66. Ju YH, Allred CD, Allred KF, Karko KL, Doerge DR, Helferich WG: **Physiological concentrations of dietary genistein dose-dependently stimulate growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in athymic nude mice.** *J Nutr* 2001, **131:**2957-2962.

국문 초록

고지방식이를 섭취한 ApoE$^{-/-}$ 마우스에서 제니스테인에 의한 비알콜성 지방간질환 완화 효과 연구

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전수경

비알코올성 지방간질환은 간세포에 지방의 과도한 축적만 일어나는 단순 지방증 (steatosis)에서 지방간염 (steatohepatitis)까지를 포함하는 일련의 질환군을 의미한다. ApoE$^{-/-}$ 마우스는 동맥경화 모델로 널리 이용되어 왔으며, 고지방식이를 섭취한 ApoE$^{-/-}$ 마우스는 비알코올성 지방간염 모델로서 제시되고 있다. 단순 지방증 수준에서 제니스테인의 저해 효과에 대한 연구는 충분히 이루어져 있으나, 염증반응을 동반하는 지방간염에서 제니스테인의 효과에 대한 연구는 미비한 실정이다. 따라서 본 연구는 ApoE$^{-/-}$ 마우스를 이용하여 제니스테인의 섭취가 비알코올성 지방 간염에 미치는 저해 효과와 관련 기전을 규명하고자 하였다. 수컷 C57BL/6 wild-type (WT) 마우스와 ApoE$^{-/-}$ 마우스를 구입하여 1주간 적응시킨 후 고지방 식이군 및 0.05% 제니스테인이 첨가된 고지방식이군으로 실험식이를 나누어 24주간 제공하였다. 혈청 중성지방, 총 콜레스테롤, alanine aminotransferase, 지질 과산화물 및 monocyte chemoattractant protein 1 의 수준은 ApoE$^{-/-}$ 마우스에서 WT 마우스에 비

해 유의적으로 높은 것이 관찰되었다. 간조직의 지질 축적과 염증 수준은 ApoE$^{-/-}$ 마우스에서 WT 마우스에 비해 유의적으로 증가됨이 관찰되었다. 이로부터 고지방식이를 섭취한 ApoE$^{-/-}$ 마우스는 지방간염 모델로서 적합하다는 것을 확인하였다. 제니스테인은 WT 마우스와 ApoE$^{-/-}$ 마우스에서 체중을 유의적으로 감소시켰다. 혈청 및 간조직 내 콜레스테롤 수준은 ApoE$^{-/-}$ 마우스에서 제니스테인의 섭취에 의해 완화되었다. 또한 ApoE$^{-/-}$ 마우스에서는 제니스테인의 섭취에 의해 혈청 MCP-1과 ALT 수준이 감소되었으며, MCP-1, tumor necrosis factor alpha, cyclooxygenase 2와 같은 항염증 유전자의 발현 수준이 감소되었다. 간 조직 내 중성지방과 peroxisome proliferator activated receptor gamma와 하위유전자인 CD36, monoacylglycerol O-acyltransferase 1과 같은 지질대사와 관련된 유전자 발현 수준은 ApoE$^{-/-}$ 마우스에서 제니스테인의 섭취에 의해 감소되었다. 위 결과들을 종합하였을 때, 제니스테인은 항산화제로 작용하여 혈청 지질 과산화물과 염증을 감소시키는 작용을 하였으며, 간으로의 지질 산화물의 유입과 더불어 지질 합성을 저해하였다. 따라서 제니스테인은 고콜레스테롤 혈증과 비만 및 비알코올성 지방간염을 포함한 대사 증후군을 개선시키는데 효과가 있을 것으로 사료된다.

주요어: 제니스테인, ApoE$^{-/-}$ 마우스, 비알코올성 지방간질환,

혈청 콜레스테롤, peroxisome proliferator activated receptor gamma

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