A THESIS FOR THE DEGREE OF
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Effect of genistein supplementation on methionine-choline deficient diet-induced nonalcoholic fatty liver disease development in *db/db* mice

제니스테인의 보충섭취가 *db/db* 마우스에서
메티오닌−콜린 결핍 식이에 의해 유도된
비알코올성 지방간질환에 미치는 영향

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Abstract

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Nonalcoholic fatty liver disease (NAFLD) occurs in a wide spectrum from simple steatosis to steatohepatitis, fibrosis and rarely, progression to cirrhosis. When *db/db* mice are fed a methionine-choline deficient (MCD) diet, significant liver fibrosis is observed as compared to other animal models. Previous studies reported that genistein has been shown to attenuate non-alcoholic steatohepatitis (NASH) induced in obese and type 2 diabetic animal models. However, there have been no studies of effect of genistein on NASH using animal model which has weight loss, anti-NASH effect of genistein is unclear. Therefore, we investigated the effect of genistein on the development of NASH induced by an MCD diet in *db/db* mice. Male C57BL/KsJ–*db/db* mice were fed a control diet or MCD diet for 6 weeks. To

investigate the effect of genistein, the additional group fed an MCD diet with 0.05% genistein. MCD diet-fed mice exhibited a significantly lower body and liver weight. And serum glucose, insulin, cholesterol and alanine aminotransferase levels were also decreased by MCD diet. MCD diet develops a higher degree of steatohepatitis corresponding with an increased oxidative stress, endoplasmic reticulum stress, hepatic steatosis, inflammation, stellate cell activation and mild fibrosis. Genistein reduced serum insulin levels, but we did not observe any inhibitory effect against hepatic steatosis by genistein supplementation. In contrast, hepatic oxidative stress and endoplasmic reticulum stress, and AMP-dependent kinase inactivation were alleviated by genistein. Genistein also down-regulated the augmented mRNA expression of genes associated with inflammatory cytokines or chemokines (tumor necrosis factor α, monocyte chemoattractant protein 1, toll-like receptor 4 and interleukin 1β) and fibrosis (transforming growth factor β1, procollagen type I and tissue inhibitors of metalloproteinase 1). In conclusion, these results suggest the genistein alleviated MCD diet-mediated NASH development by suppressing lipid peroxidation, inflammation and even liver fibrosis in *db/db* mice.

Key words: *db/db* mouse, fibrosis, genistein, methionine-choline deficient diet, non-alcoholic steatohepatitis

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List of Abbreviations

ALT : alanine aminotransferase

AMPK : AMP-dependent kinase

BiP : immunoglobulin heavy-chain binding protein

CHOP : C/EBP homologous protein

COL1A1 : collagen, type I, alpha 1

CYP2E1 : cytochrome P450 2E1

ER : endoplasmic reticulum

HO-1 : heme oxygenase 1

HOMA-IR : homeostasis model assessment-insulin resistance

HSC : hepatic stellate cells

IL-1β : interleukin-1 beta

MCD : methionine-choline deficient

MCP-1 : monocyte chemoattractant protein 1

MCS : methionine-choline sufficient

NAFLD : non-alcoholic fatty liver disease

NASH : non-alcoholic steatohepatitis

PPARα : peroxisome proliferator-activated receptor alpha

ROS : reactive oxygen species

TBARS : thiobarbituric acid reactive substances

TGF-β : transforming growth factor beta

TIMP-1 : Tissue inhibitor of metalloproteinase 1

TLR4 : Toll-like receptor 4

TNFα : tumor necrosis factor alpha

UPR : unfolded protein response

XBP-1 : X-box binding protein 1

Introduction

Nonalcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is characterized by the intracellular accumulation of lipids in the liver (over 5-10% by weight) [1], and is a range of disease states, from steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH; steatosis with inflammatory changes) followed by progression to fibrosis, cirrhosis and hepatocellular carcinoma [2]. NASH is the more pathologically advanced stage of the disease and is characterized by increased hepatocellular damage (ballooning of hepatocytes), chronic liver inflammation, and fibrosis [3]. NAFLD tends to develop in patients who are obese or have diabetes. Most obese adults have hepatic steatosis and at least one-third of these individuals will eventually develop worsening NAFLD [4, 5], therefore the prevalence of NAFLD will likely rise with obesity rates. There are current treatment strategies for NAFLD. Treatment strategies, in particular approaches that stop the progression of the disease, are thus of eminent importance. Nevertheless, effective weight loss, the initial step recommended for NASH therapy, is not durable and cannot be achieved in the majority of patients. Several drug therapies have been directed against the factors that play a role in the pathogenesis of NASH. However, there are no ideal

pharmacological treatments [6].

Transition of steatosis to steatohepatitis

Fatty liver is the basic feature of NAFLD and NASH. Triglycerides are the main types of lipids stored in the liver of patients with NAFLD. The toxic lipids in NASH and the non-toxic lipids in simple steatosis might be different [7]. Cell injury may occur when the capacity of hepatocytes to safely store fat is overwhelmed by continued uptake [8], local synthesis, or impaired egress of fatty acids [9]. These fatty acids then become toxic to the cell in a pathobiological process termed lipotoxicity. Lipotoxicity can cause cell death by the direct effects of lipid mediators on apoptosis. Alternatively, liberation of oxidized lipids and their peroxidation products (chemotactic aldehydes and organic acids) might be instrumental in recruiting and maintaining the inflammatory response that characterizes NASH. The fatty liver is predisposed to forms of injury that involve oxidative stress. Steatosis could provide the setting, first hit, for NASH, but a second hit may be needed to cause cellular injury or recruit inflammation (**Fig. 1**) [10]. The “two-hit” model of NASH pathogenesis was proposed in response to the experimental observations that endotoxin causes focal cytolysis and inflammation with amplified release of tumor necrosis factor alpha (TNFα) in rodent models of

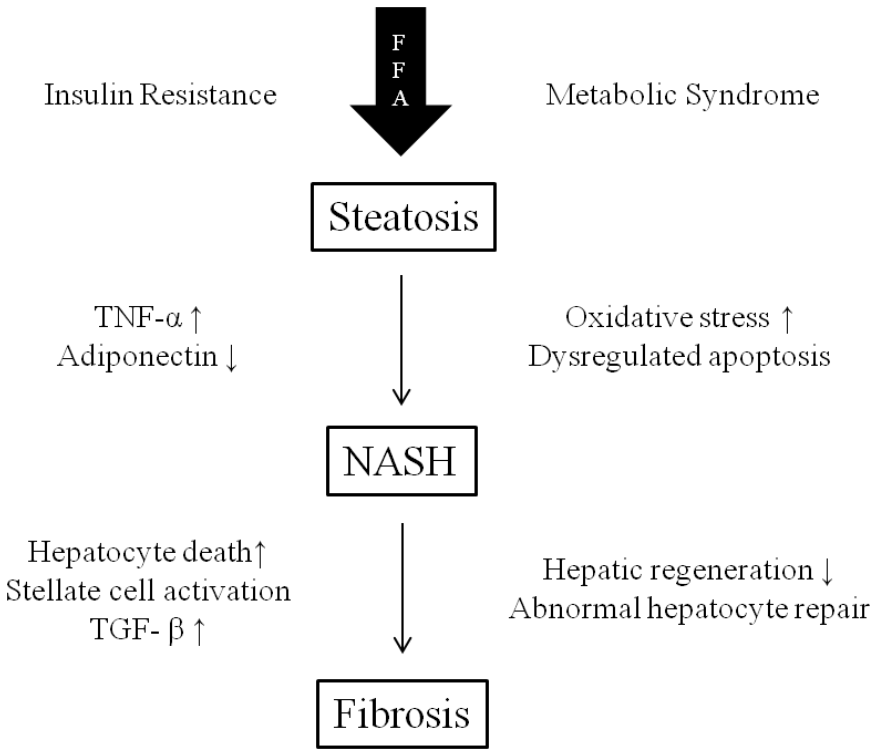


Figure 1. "Two-hit" hypothesis of NAFLD

Dysregulated free fatty acid (FFA) metabolism (the first hit) results in lipid accumulation in the liver, producing steatosis. Inflammatory mediators, reactive oxygen species, and abnormal apoptotic mechanisms serve as second hits that result in superimposed inflammation (NASH). The interplay between inflammatory mediators and the activation of stellate cells can lead to fibrosis.

steatosis [11], and the livers of patients with NASH as well as animal models of metabolic steatohepatitis over-express cytochrome P450 2E1 (CYP2E1) [12], a microsomal fatty acid oxidase that generates reactive oxygen species. Oxidative stress, TNFα, other cytokines, and chemokines are all present in NASH, but the ways in which they initiate or perpetuate steatohepatitis remain uncertain. Recent data indicate that oxidative stress may recruit inflammation via activation of NF-κB [13].

Fibrogenesis

Liver fibrosis is a consequence of the accumulation of extracellular matrix materials, including type 1 collagen, which are generated by activated hepatic stellate cells (HSC) and hepatic myofibroblasts. When liver injury takes place, HSCs undergo activation and change their phenotype into myofibroblast-like cells [14]. Activated Kupffer cells, infiltrating monocytes, activated and aggregated platelets, and damaged hepatocytes are the sources of platelet-derived growth factor and transforming growth factor beta (TGF-β), which trigger the initiation of intracellular signaling cascades that lead to the activation of HSCs. Other factors such as mitochondrial injury [15] and microvascular injury [16] also might mediate or exaggerate the pathogenic processes that lead to hepatic fibrosis of cirrhosis.

Oxidative stress could be directly involved in fibrogenesis, as shown by the protective effects of vitamin E in a nutritional model of NASH [17]. Alternatively, mobilization of profibrogenic cytokines (connective tissue growth factor, TGF-β) could be involved, liberated in response to hyperglycemia or necroinflammatory change [10]. Many of these processes reflect interactions between host genes and environmental factors in complex, interactive pathogenic networks. For example, feeding obese *fa/fa* rats a high-fat diet was sufficient to cause oxidative stress associated with steatohepatitis and periportal fibrosis [18].

Methionine-choline deficient (MCD) diet

The pathophysiological mechanisms underlying progression from steatosis to NASH remains obscure, due in part to lack of appropriate experimental animal model. The methionine-choline deficient (MCD) diet has been used most often to produce a dietary model of NASH in animals. The MCD diet induces significantly greater reactive oxygen species (ROS) production, hepatic DNA damage and apoptotic cell death than many of the other mouse NAFLD models [19] and accumulated data reveal that this model produces steatohepatitis, inflammation, and fibrosis that is histologically similar to human NASH [12, 20], although this model is limited by the lack of most extra-hepatic metabolic abnormalities, which

is typically seen in humans with NASH. Increased fatty acid uptake and decreased very low-density lipoprotein secretion were considered to be important mechanisms by which the MCD diet promoted intrahepatic lipid accumulation in this model [21]. This may explain low levels of total cholesterol and triglycerides in blood. Several attempts to overcome these metabolic obstacles include feeding an MCD diet to genetically obese and diabetic mice. Although an MCD diet causes weight loss and improves insulin resistance even in *db/db* mice, *db/db* mice fed an MCD diet develop greater pericellular fibrosis as well as steatosis, inflammation, and ballooning degeneration, which makes these animal models to be better fit to investigate the pathogenesis of human NASH [22]. One possible explanation for weight loss by feeding the MCD diet could be MCD-induced suppression of stearoyl-coenzyme A desaturase-1 in the liver, which likely contributes to hypermetabolism and weight loss, and induces hepatic steatosis [23]. Another possible explanation could be low food intake.

Other animal models of NAFLD/NASH

The major approaches to modeling the induction of NAFLD/NASH in rodents can be divided into two broad categories, genetic models or dietary models. Sterol regulatory element binding protein-1c (SREBP-1c), a lipogenic transcription factor,

transgenic mice creates congenital lipodystrophy in which severe insulin resistance and diabetes [24]. In these mice, decreased fat tissue with lipid accumulation in the liver is observed and marked hepatic steatosis occurs. *Ob/ob* mice, lacking leptin, are obese, hyperphagic, insulin resistant and develop hepatic steatosis and type 2 diabetes. Unlike the human NAFLD patients, *ob/ob* mice do not spontaneously progress from steatosis to steatohepatitis [25]. Two methods are commonly used to induce steatohepatitis in this model, either a low-dose lipopolysaccharide or administration of the MCD diet [26]. They are protected against fibrosis, a phenomenon which led to the characterization of leptin as an essential mediator of hepatic fibrogenesis [27]. Peroxisome proliferator-activated receptor-α (PPAR-α) is a key regulator of genes involved in peroxisomal, mitochondrial and microsomal fatty acid oxidation in the liver. PPAR-α knockout mice are observed in the high fat (HF) diet model [28]. HF diet of dietary models showed elevated plasma insulin levels reflecting insulin resistance [29]. Like human NASH, HF diet developed oxidative damage in the liver. In general, compared with the MCD model, the degree of liver injury in the HF model is less severe [30]. Experimental animals fed a fructose-enriched diet are recognized as good models of metabolic syndrome. Rats fed a fructose-enriched diet developed macrovesicular and microvesicular steatosis in the liver [31]. Recently, it was reported that c57BL/6 mice fed a high-fat high-carbohydrate diet and provided with drinking water containing 55%

fructose for 16 weeks developed a NASH with significant fibrosis as well as obesity [32].

Oxidative stress

Although the exact pathogenesis of NAFLD is yet to be understood, previous studies have demonstrated oxidative stress is a major contributor in the pathogenesis of NAFLD and have focused on strongly related markers such as lipid peroxidation, ROS production, insulin resistance, cholesterol accumulation, and secretion of adipokines [33, 34]. Marker is used to assess oxidative stress including malondialdehyde (MDA), which is product of lipid peroxidation [35]. Under normal conditions, hepatic aerobic metabolism involves a steady-state production of pro-oxidants such as ROS, which are balanced by a similar rate of their consumption by anti-oxidants [36]. Limited anti-oxidant defenses contribute to the processes of both NASH and hepatocarcinogenesis. [37] High serum free fatty acids (FFAs) level, providing substrates for triglyceride formation and subsequent progression of the disease in the liver [38]. It is suggested that increased accumulation of liver triglycerides leads to increased oxidative stress in the hepatocytes of animals and humans [39]. Recent evidence has shown increased oxidative stress due to increased fat influx into the liver [40]. There is intense

interest in the role of transcription factors in the pathogenesis of NASH, particularly PPARα. PPARα activation increased fatty acid oxidations, reduces adiposity, inhibition of vascular inflammation and improves hepatic steatosis [20]. Kupffer cells and HSC play significant roles in the progression of chronic liver inflammation and fibrosis progression [15]. Excess fatty acid accumulation in hepatocytes induces oxidative stress from not only mitochondria but also peroxisome or microsome. These cytotoxic ROS and lipid peroxidation products can diffuse into the extracellular space affecting Kupffer cells and HSC. The cellular oxidative stresses form hepatocytes and the direct uptake of free fatty acids of free cholesterol in Kupffer cells induce the activation of NF-κB, which induces the synthesis of TNFα and several pro-inflammatory cytokines [41].

Endoplasmic reticulum (ER) stress

The endoplasmic reticulum (ER) is an essential organelle which provides a specialized environment for the production and post-translational modifications of secretory and membrane proteins [42, 43]. When protein folding in the ER is inhibited, signal transduction pathways, which increase the biosynthetic capacity and decrease the biosynthetic burden of the ER to maintain the homeostasis of this organelle, are activated. These pathways are called the unfolded protein response

(UPR) [44]. Certain pathological stress conditions disrupt ER homeostasis and lead to accumulation of unfolded or misfolded proteins in the ER lumen, so called ER stress. The UPR promotes cell survival by activating genes and proteins that halt further ER protein accumulation. Three UPR transducers (Inositol-requiring enzyme 1, IRE1; Pancreatic endoplasmic reticulum kinase, PERK; Activating transcription factor 6, ATF6) and one master regulator, immunoglobulin heavy-chain binding protein (BiP), are central to this process. In the normal state, UPR transducers are bound by BiP. In response to ER stress, dissociation of BiP initiates activation of these proteins. IRE1 forms a dimer and autophosphorylates, resulting in mRNA splicing of the X-box-binding protein 1 (XBP-1). Activated XBP-1 up-regulates ER chaperone genes such as BiP. If the survival response is inadequate, a cell signaling cascade do induce apoptosis is initiated, which includes activation of C/EBP homologous protein (CHOP). Recently, C/EBP family of transcription factors have emerged as important regulators of hapatic lipid metabolism. CHOP, a transcription factor that potentiates apoptosis [45]. In the previous study, mice fed the MCD diet showed a significant 1.5-fold increase in CHOP protein levels [46], confirming that the MCD diet activates down-stream mediators of the ER stress pathway. Bcl-2 family members including anti-apoptotic proteins (e.g. Bcl-2) and pro-apoptotic proteins (e.g. Bax) could be major candidates for JNK regulation of cell apoptosis [47, 48]. Hepatocyte apoptosis in addition to oxidative stress could

be a key component in the pathogenesis of NASH. Hepatocytes, like other secretory cells, are rich in ER. Because of their high protein synthesizing capacity it is easy to postulate that UPR/ER stress response plays an important role either in preventing of mediating pathological changes in various liver diseases. The ER plays a significant role in fatty acid synthesis and cholesterol metabolism. The relationship between ER stress and fatty liver is a bilateral one. Steatosis has been shown to promote ER stress [49, 50]; conversely, ER stress leads to steatosis [51-54]. Several studies have confirmed the activation of the ER stress pathway in the steatotic liver of various animal models [46, 52, 55, 56] or in humans [57, 58]. Thus, the hepatic activation of ER stress suggests a role of this cellular pathway in the onset of hepatic steatosis.

Genistein

Genistein (4', 5, 7-trihydroxyisoflavone), a phytoestrogen found in soybeans, has a variety of pharmacological features. Phytoestrogens have anti-oxidant features and consumption of soy products and isoflavones that are rich in phytoestrogens have proved to bear numerous favorable effects on various disease [59]. Genistein displays its anti-oxidant action in several ways by the Fe^{2+}-ADP complex [60] and by low density lipoprotein oxidation mediated by copper and peroxyl radicals [61].

The anti-proliferative action of genistein includes prevention of DNA mutation, reduction in cancer cell proliferation, and prevention of oxidative DNA damage. Besides anti-oxidant property, other reported effects of genistein's anti-inflammatory effect, such as property to inhibit tyrosine kinase activity. Tyrosine kinases are known to play important roles in cellular proliferation and differentiation [62]. Genistein has been shown to be effective in preventing lipopolysaccharide induced NF-κB activation as well as NF-κB dependent cytokine such as IL-6, IL-8 and TNFα production *in vitro* [63]. In previous studies isoflavones, especially genistein, are related with glucose homeostasis and lipid metabolism and on hormones controlling their metabolism [64, 65]. In addition, genistein has been shown to prevent progression to NASH in a rat fed with a high fat diet [66]. By increasing gene expression for enzymes of fatty acid oxidation and inhibiting the expression of those involved in lipogenesis, genistein lower serum lipid concentrations and fat storage in the body [64, 65, 67, 68]. Likewise, most previous studies reported inhibitory effect of genistein against NAFLD development in obese animal models, an animal model featured with distinct patterns of tissue-specific lipid accumulation between in liver and in other tissues may provide a better understanding of the role of genistein in the regulation of hepatic and peripheral lipid accumulation and inflammatory responses. Genistein also shows anti-fibrotic effects by decreasing *in vitro* collagen synthesis induced by

TGF-β1 [69]. The study revealed that genistein affects proliferation of hepatic stellate cells that are responsible from hepatic fibrogenesis and consequently displays anti-fibrotic effects. But there is no study which confirms the anti-fibrotic effect of genistein in diet-induced NASH.

Aim of this study

Thus, the objective of this study was to determine whether dietary genistein would alleviate NASH development by suppressing lipid peroxidation, inflammation and even liver fibrosis in *db/db* mice fed an MCD diet apart from anti-obesity effect.

Materials and Methods

Animals and diets

Male C57BLKS/J-*db/db* mice, 5 weeks of age, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). After 2 weeks of acclimatization, the treatment was started considering the event when the amount of fasting blood glucose of mice is more than 300 mg/dl as the insulin resistance. The mice were randomly divided into three groups : Group 1 (n=10) was fed a methionine and choline sufficient diet (MCS diet), Group 2 (n=10) received an MCD diet, Group 3 (n=10) fed a special diet that was MCD diet supplemented with genistein (0.5g/kg diet; ChromaDex Inc., USA) for 6 weeks. The MCS and MCD diet were obtained from Dyets Inc. (#518811 and 518810, respectively; USA). The composition of the diets is given in **Table 1**. All Mice were allowed food and water *ad libitum*. Animals were housed two mice per cages and maintained in a temperature (22 ± 3℃) and humidity (50 ± 10%)-controlled room with a 12 h dark-light cycle. All animal experiments were permitted by the Institute of Laboratory Animal Resources of the Seoul National University (permission number SNU-100524-5), and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Seoul National University.

Table 1. Composition of the experimental diets

Ingredient	MCS diet	MCD diet	MCD+GEN diet
		(g / kg)	
L-amino acid[a]	142	142	142
Cornstarch	100	100	100
Dextrin	100	100	100
Sucrose	398	408	408
Cellulose	50	50	50
Corn oil	50	50	50
Salt mix	35	35	35
Sodium bicarbonate	4.3	4.3	4.3
Vitamin mix	10	10	10
Primex[b]	100	100	100
Ferric citrate, U.S.P	0.33	0.12	0.12
L-methionine	1.7	-	-
Choline chloride	8.0	-	-
Genistein	-	-	0.5

[a] Total L-amino acid (without L-methionine)

[b] Hydrogenated vegetable oil

Body weight and fasting blood glucose

The animals were supplemented for a period of 6 weeks. During the experiments, food consumption was monitored at 4 times of a week. Body weights were recorded and blood glucose levels were measured with a glucose meter (Accu-chek® Go, Roche, USA) after 6 h fasting every week.

Blood and tissue collection

At the end of the study, after the mice fasted for 16 h and sacrificed. Mice were anesthetized via intramuscular injection of tiletamine-zolazepam (Zoletil 50, Virbac Laboratories, France) at a dose of 12.5mg/kg. Blood was collected by cardiac puncture and centrifuged for 20 min, at 3,000 rpm at 4 ℃. Under the same condition of anesthesia, liver, and adipose tissue (epididymal fats) were removed and then washed with phosphate-buffered saline (PBS) and weighed. Serum and tissues were quickly frozen in liquid nitrogen and stored at -80℃ deep freezer until further analysis. Part of liver was fixed in 10% formalin for histological analysis.

Serum biochemical analyses

Serum was analyzed using commercial colorimetric assay kits (Asan Pharmaceutical Co., Korea) for glucose, triglyceride, total cholesterol and alanine

aminotransferase (ALT) according to the manufacturer's protocol. And all samples were assayed in duplicate. Serum insulin levels were determined using an insulin ELISA kit (Millipore, USA). The insulin resistance index was assessed by homeostasis model assessment-insulin resistance (HOMA-IR) using the following formula: HOMA-IR = [fasting glucose (mM/L) × fasting insulin (μU/mL)]/22.5.

Hepatic biochemical analyses

Hepatic total lipids were extracted according to the method of Folch et al [70]. 50mg liver tissues were homogenized with chloroform : methanol (2 : 1, *v/v*) and added 0.88% KCl. Then the extract was centrifuged for 15 min at 1,000xg at 4℃ and evaporated to dryness under nitrogen. The residue was suspended in the isopropanol and kept in -80℃ deep freezer before analyzed. Liver Triglyceride and cholesterol concentrations were determined by the same commercial kits used in serum (Asan Pharmaceutical Co.) biochemistry. Lipid peroxides were assessed as thiobarbituric acid reactive substances (TBARS) according to the method developed by Ohkawa et al [71]. Liver tissues (50 mg) were homogenized in 0.5 ml homogenizing buffer (10 %, *w/w*) consisting of 154 mM KCl, 50 mM Tris-HCl, 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 600xg (4℃) for 10 min to obtain the supernatant. For TBARS, 10% homogenate (80 μl) was mixed with

8.1% sodium dodecyl sulfate (SDS), 20% acetic acid and 0.8% TBA solution and boiled at 95℃ for 60 min. After cooling, n-butanol and pyridine (15 : 1, *v/v*) were added to the reaction mixture, and the mixture was centrifuged at 4,000 rpm for 20 min. The absorbance of the butanol layer (the supernatant) was measured at 532 nm using 1,1,3,3-tetraethoxypropane as a standard. All samples were assayed in duplicate. The hepatic lipid peroxide level was expressed as MDA equivalents per milligram of protein. The protein content of the homogenate was measured with a protein assay kit (Bio-Rad, USA).

Liver histology examination

Formalin-fixed liver tissue was processed into 4-μm-thick paraffin sections and stained with hematoxylin and eosin (H&E) for histological evaluation. Liver fibrosis was assessed with Masson's trichrome staining for collagen fibers.

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 (Sigma, USA)]. After

centrifugation for 30 min at 10,000xg at 4℃, the supernatant was transferred to new tube and kept in -80℃ deep freezer before analyzed. The protein content was measured in all supernatants using protein assay kit (Bio-Rad, USA). Proteins in 10% homogenates samples were loaded into the lanes of an SDS-PAGE gel, separated on an either 8% gel [Bip], 10% gel [AMPK], 12% gel [CHOP], 15% gel [Bax] with a Tris-glycine running buffer system and then transferred to PVDF membrane using a semi-dry electrotransferring unit (Bio-Rad, USA) at 15 V for 60 min. After the membranes had been blocked 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween-20 containing 5% nonfat milk or bovine serum albumin at room temperature. The specific antibodies used are as follows: anti-AMP kinase α (AMPKα; #2532, Cell Signaling, USA), anti-p-AMPKα (#2531s, Cell signaling), anti-C/EBP homologous protein (CHOP; sc-575, Santa Cruz Biotechnology, USA), anti-KDEL (ADI-SPA-827, Enzo Life Sciences, USA), anti-Bax (#2772, Cell signaling) or anti-70-kDa heat shock cognate protein (HSC70; #sc-7298, Santa Cruz Biotechnology). Anti-HSC70 or anti-AMPK were used as a control protein. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs were used as secondary antibodies. The immunoreactive protein bands were visualized by Immobilon western chemiluminescent using HRP substrate (Millipore, USA) with exposure to FUJI super RX X-ray film. The intensities of band were quantified with the Quantity one software (Bio-Rad).

Total RNA extraction and semi-quantitative PCR analyses

Total RNA of liver tissues was isolated using RNAiso reagent (TaKaRa, Japan). Purified RNA (2 μg) was reverse transcribed with superscript® Ⅱ Reverse Transcriptase (Invitrogen, USA) and then cDNA was synthesized. The target cDNA was amplified using the following primers. Xbp-1 [72], β-actin [73] (Bioneer, Korea). The primers for Xbp-1 generate cDNA products of the unspliced 480 bp Xbp-1 mRNAs and spliced 454 bp Xbp-1 mRNAs respectively. These unspliced fragments were further digested by PstⅠ(promega, USA). cDNA products from the unspliced mRNA yielded two short fragments (291 bp and 189 bp) after digestion. Expression of β-actin was used as an internal standard. Amplified products were separated on an agarose gel (1.5%) and visualized with ethidium bromide staining.

SYBR green real-time PCR analyses

Hepatic mRNA levels were analyzed by qPCR using a StepOne™ Real Time PCR System (Applied Biosystems, USA) using the SYBR® Green PCR Master Mix (Applied Biosystems). To determine gene expression using SYBR green, reactions were carried out in a 20 μl reaction volume containing 2 μl of reverse-transcribed cDNA, 10.8 μl of SYBR® Green PCR Master Mix, 0.4 μl of 10 μM reverse primer,

and 7.2 μl of Ultrapure^{TM} Distilled Water (Invitrogen). The primer sequences are described in **Table 2.** Conditions for all qPCR reactions were 95℃ for 10 min to initiation and then run for 40 cycles at 95℃ for 15 sec, 60℃ for 1 min and 95℃ for 15 sec. Mouse ribosomal protein L19 (RPL19) was used as a reference gene and relative gene expression levels were analyzed using the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

All data were analyzed using SPSS software (ver. 21.0, SPSS Inc., USA). For all experiments, one-way ANOVA followed by Duncan's multiple range test or Student *t*-test were employed to assess the statistical significance. Data were expressed as means ± SEM and differences were considered statistically significant at $P < 0.05$.

Table 2. Primer sequences for qPCR and semi-quantitative PCR

Primer	Forward(5'-3')	Reverse(5'-3')
	qPCR	
COL1A1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
HO-1	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
MCP-1	CCAGCACCAGCACCAGCCAA	TGGGGCGTTAACTGCATCTGGC
PPARα	CCTCAGGGTACCACTACGGAGT	GCCGAATAGTTCGCCGAA
RPL19	TCAGGCTACAGAAGAGGCTTGC	ATCAGCCCATCCTTGATCAGC
TGFβ	CACCGGAGAGCCCTGGATA	TGTACAGCTGCCGCACACA
TIMP1	TGGGAAATGCCGCAGATATC	TGGGACTTGTGGGCATATCC
TLR4	AGGAAGTTTCTCTGGACTAACAAGTTTAGA	AAATTGTGAGCCACATTGAGTTTC
TNFα	GGCTACAGGCTTGTCACTCGA	CACGCTCTTCTGTCTACTGAA
	Semi-quantitative PCR	
XBP-1	AAACAGAGTAGCAGCGCAGACTGC	TCCTTCTGGGTAGACCTCTGGGAG
Beta-actin	TGACCCAGATCATGTTTGAGACC	CCATACCCAAGAAGGAAGGC

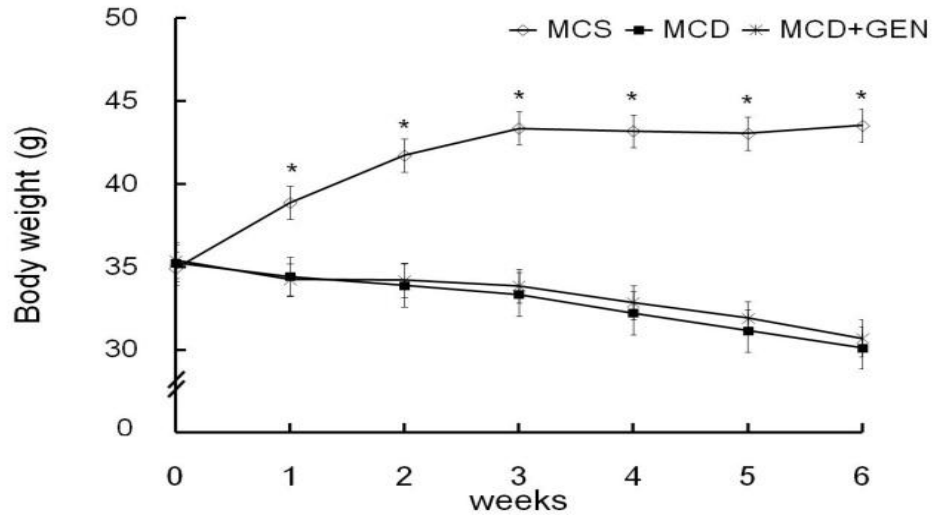
Results

Effect of genistein on changes of body weight and fasting blood glucose during the experimental period

The changes of body weight are shown in **Fig. 2A**. We observed the gradual weight decreased in *db/db* mice fed an MCD diet. This weight loss was apparent immediately after starting the diet, and retained until 6 weeks. MCD diet-induced weight loss may be partly attributable to metabolic rate was increased [23]. Mice fed a diet supplemented with genistein do not have any effect on changes of body weight compared with mice fed an MCD diet.

All *db/db* mice were diabetic, when experiment began, as indicated by the blood glucose levels (≥ 300 mg/dl). MCD diet decreased blood glucose levels significantly compared with MCS diet at 1 week after starting the diet and this tendency stabilized after 2 weeks. Genistein supplementation does not affect blood glucose level (**Fig. 2B**).

(A)



(B)

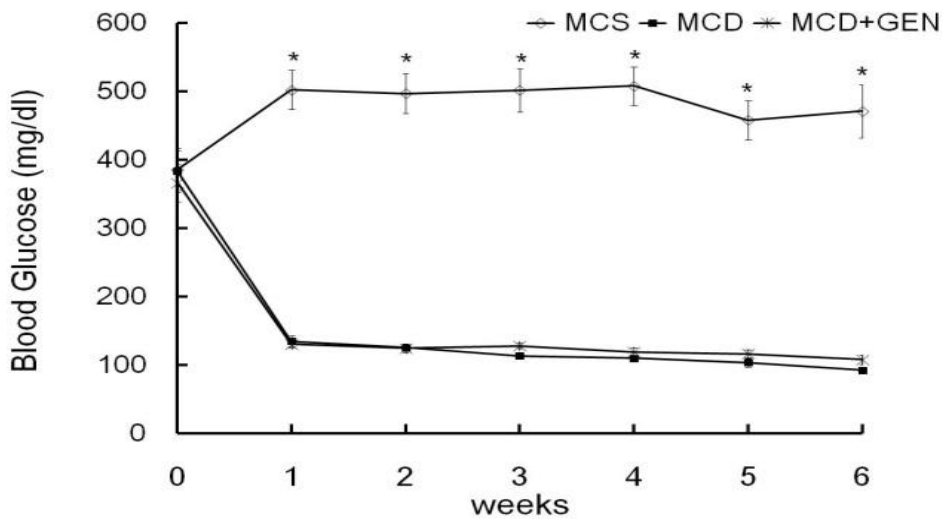


Figure 2. Effect of genistein on changes of body weight and fasting blood glucose

Changes of **(A)** body weight and **(B)** fasting blood glucose levels for 6 weeks in *db/db* mice fed either MCS, MCD or MCD+GEN diet. Each bar represents the mean ± SEM (n = 10). * MCS *vs.* MCD and MCD + GEN ($P < 0.05$).

Effect of genistein on final body weight, organ weight and food consumption

The *db/db* mice fed an MCS diet were significantly more obese than MCD diet. Six-weeks of MCD diet reduced about 17% of body weight in *db/db* mice (**Table 3**). There were no statistical differences in the initial body weights of all groups. Food consumption in *db/db* mice fed an MCD diet was significantly decreased compare with MCS diet group, it indicates that MCD diet eliminated baseline hyperphagia in *db/db* mice.

The liver weight and relative liver weight of *db/db* mice fed an MCS diet were significantly higher than *db/db* mice in both MCD and MCD+GEN diet. Although MCD diet led to decrease liver weight of *db/db* mice, genistein supplementation does not affect liver and relative liver weight.

The epididymal fat weights were also significantly decreased by MCD diet. There are no significantly differences in relative epididymal fat weights between MCS and MCD groups. Genistein supplementation does not affect epididymal fat weights, however, relative epididymal fat weights were significantly increased compared to MCD groups.

Table 3. Effect of genistein on body and organ weight and food consumption

	MCS	MCD	MCD+GEN
Baseline body weight (g)	37.89 ± 0.93^{NS}	35.23 ± 1.13	35.39 ± 1.09
Final body weight (g)	42.0 ± 1.4^{b}	29.1 ± 1.2^{a}	29.8 ± 1.1^{a}
Liver weight (g)	3.47 ± 0.26^{b}	1.62 ± 0.10^{a}	1.69 ± 0.10^{a}
Relative liver weight (% of body weight)	8.19 ± 0.38^{b}	5.55 ± 0.27^{a}	5.64 ± 0.19^{a}
Epididymal fat (g)	2.27 ± 0.10^{b}	1.47 ± 0.08^{a}	1.69 ± 0.08^{a}
Relative epididymal fat weight (% of body weight)	5.47 ± 0.21^{ab}	5.01 ± 0.10^{a}	5.67 ± 0.09^{b}
Food consumption (g/day)	6.87 ± 0.28^{b}	3.27 ± 0.21^{a}	3.24 ± 0.13^{a}

Results are given as means ± SEM (n = 10). Means with different superscripts are significantly different by ANOVA ($P < 0.05$)

Effect of genistein on serum biochemical analyses

As shown in **Table 4**, MCD diet did significantly lower serum glucose and insulin levels, and HOMA-IR compared with MCS diet. Moreover, supplementation of genistein significantly reduced serum insulin levels and HOMA-IR (P = 0.014, *t*-test). Previous studies report that MCD diet reduced blood glucose and insulin levels and alleviated peripheral insulin resistance [74, 75].

Although MCD feeding significantly reduced serum cholesterol levels, no difference in serum triglyceride level was observed in *db/db* mice fed an MCD diet. Genistein supplementation does not affect serum cholesterol and TG levels.

Serum ALT levels which has been identified as a marker of liver injury, were significantly increased in *db/db* mice fed an MCD diet compared to MCS diet, but there were no significant effect of genistein on serum ALT levels.

Table 4. Effect of genistein on changes of fasting serum biochemical parameters

	MCS	MCD	MCD+GEN
Glucose (mg/dL)	765.9 ± 74.7^{b}	105.4 ± 7.1^{a}	127.0 ± 14.4^{a}
Insulin (ng/mL)	1.59 ± 0.24^{c}	0.92 ± 0.13^{b}	0.39 ± 0.09^{a}
HOMA-IR	67.86 ± 12.00^{a}	5.63 ± 1.04^{b}	2.48 ± 0.34^{b}
Triglyceride (mg/dL)	122.8 ± 14.0	121.4 ± 12.8	108.4 ± 2.9
Cholesterol (mg/dL)	230.9 ± 21.9^{b}	126.7 ± 4.7^{a}	128.3 ± 10.7^{a}
ALT (IU/L)	180.1 ± 23.6^{a}	332.1 ± 43.9^{b}	296.8 ± 9.8^{b}

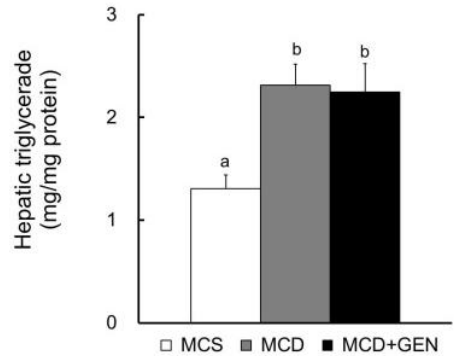
Results are given as means ± SEM (n = 7). Means with different superscripts are significantly different by ANOVA ($P < 0.05$)

Effect of genistein on hepatic lipid profiles and liver histology

As shown in **Fig. 3A**, hepatic triglyceride of *db/db* mice fed an MCD diet significantly increased compared to MCS diet. The supplementation of a MCD diet with genistein did not alleviate the accumulation of hepatic triglyceride in *db/db* mice (**Fig. 3B**). There is no significant difference in hepatic cholesterol levels among the groups.

The microscopic examination of H&E staining of liver tissues was consistent with hepatic lipid biochemical characteristics (**Fig. 4**). We observed the microvesicular steatosis in *db/db* mice fed an MCS diet. Furthermore, exacerbated macrovesicular steatosis as well as inflammation was induced by MCD diet, which were less observed in *db/db* mice fed a genistein-supplemented diet.

(A)



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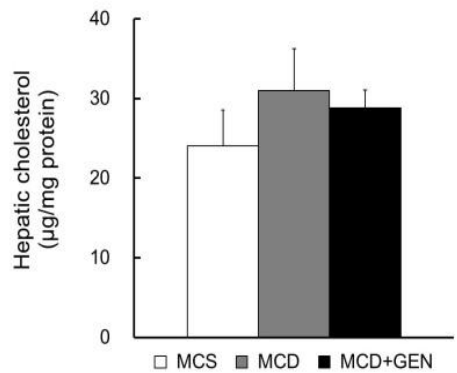


Figure 3. Effect of genistein on hepatic triglyceride and total cholesterol levels

(A) Hepatic triglycerides and **(B)** hepatic total cholesterol concentration in *db/db* mice fed either MCS, MCD or MCD+GEN diet. Data are presented the means ± SEM (n = 7). Bars with different superscripts are significantly different by ANOVA ($P < 0.05$).

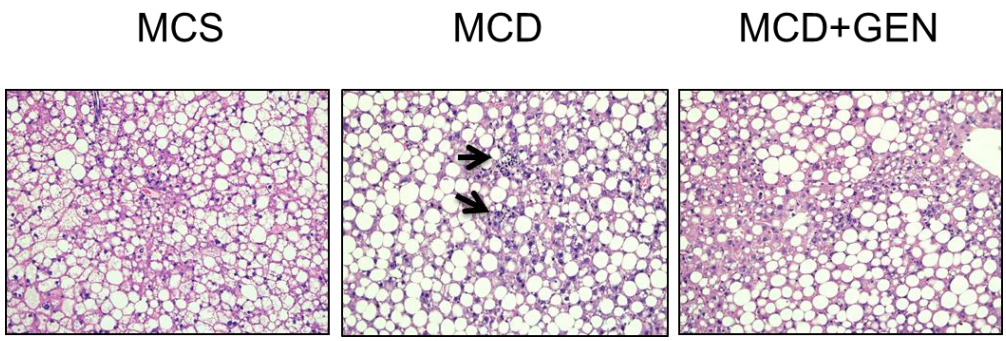


Figure 4. Representative H&E staining of liver tissue sections

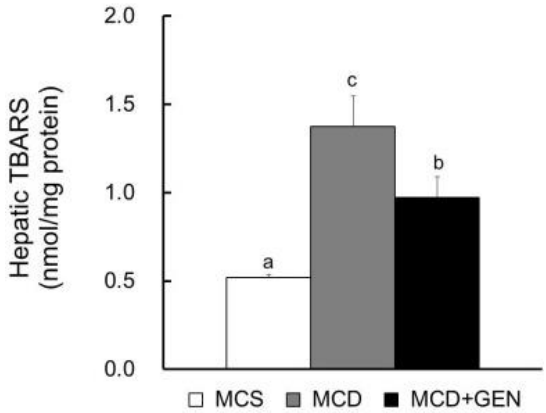
Representative H&E staining of liver tissue sections (n = 4). Inflammatory foci are indicated with black arrows.

Effect of genistein on oxidative stress

To evaluate hepatic oxidative stress, we measured hepatic MDA levels, an indicator of lipid peroxidation. Hepatic TBARS levels showed a tendency to increase in *db/db* mice fed an MCD diet compared to MCS diet. Genistein supplementation significantly decreased hepatic TBARS (**Fig. 5A**). The increased generation of ROS by microsomal CYP2E1 enzyme has been proposed as an initiator of oxidative stress associated in MCD diet-mediated steatohepatitis in mice [7].

Accordingly, the mRNA levels of heme oxygenase (HO-1) were significantly induced in mice fed an MCD diet (**Fig. 5B**). HO-1, the rate-limiting enzyme in heme catabolism, has been reported to have potential anti-oxidant properties. It is known to be induced in response to NAFLD and other liver injuries. Genistein supplementation significantly reduced the mRNA levels of HO-1.

(A)



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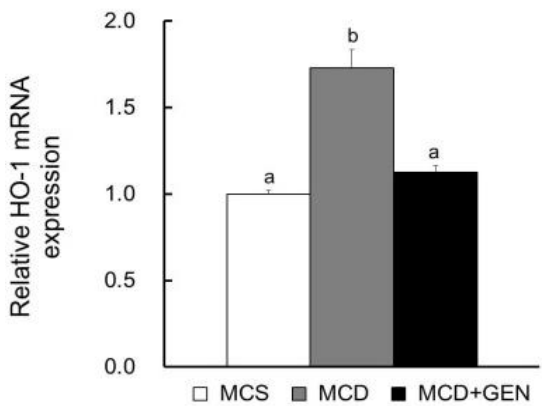


Figure 5. Effect of genistein on hepatic lipid peroxidation and HO-1 mRNA expression

(A) Hepatic TBARS levels (n = 5). **(B)** Relative mRNA expression of HO-1/RPL19 was determined by qPCR (n = 3-4). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

Effect of genistein on ER stress

ER stress in addition to oxidative stress could be a key component in the pathogenesis of NASH and has been shown to be induced in an MCD diet-induced NASH model. To determine the effects of genistein on ER stress, we examined the cellular markers of ER stress including BiP, CHOP and Bax proteins [76]. BiP, master regulator of UPR, initiate activation of UPR transducers. CHOP causes down-regulation of the anti-apoptotic protein. Pro-apoptotic protein Bax levels increased with ER stress. As shown in **Fig. 6**, protein expression levels of BiP, CHOP and Bax were significantly increased in *db/db* mice fed an MCD diet compared to that in *db/db* mice fed an MCS diet. Genistein supplementation significantly alleviated the expression levels of these proteins induced in mice fed an MCD diet.

We also measured spliced form of XBP-1 mRNA levels. ER stress induced splicing of XBP-1. Significant induction of spliced form of XBP-1 mRNA in mice fed an MCD diet. The suppressive effect of genistein was significant (**Fig. 7**).

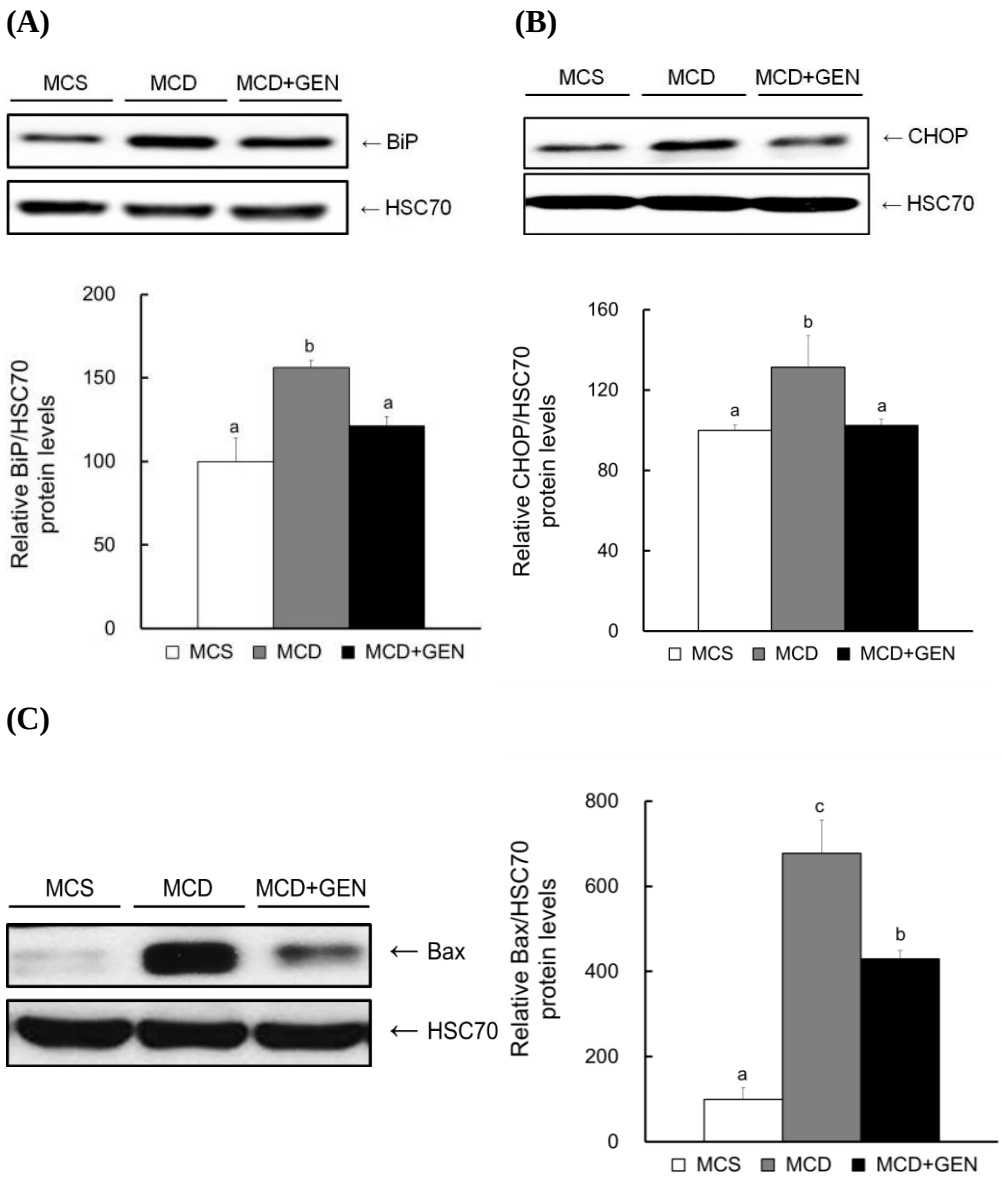


Figure 6. Effect of genistein on relative expression of proteins related with ER stress

Relative **(A**) BiP/HSC70, **(B)** CHOP/HSC70, **(C)** Bax/HSC70 protein levels were determined by immunoblotting (n = 3). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

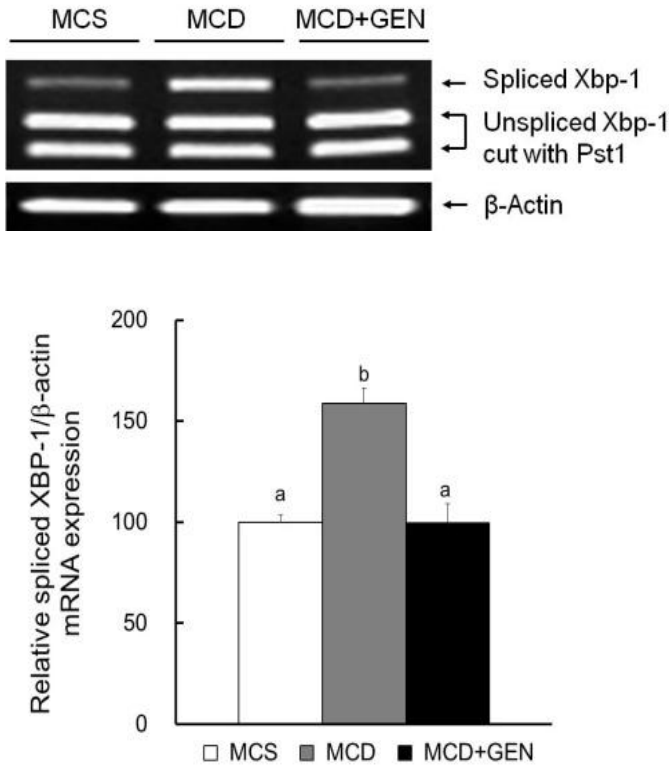


Figure 7. Effect of genistein on relative expression of Xbp-1 levels related with ER stress

Relative mRNA expression levels of spliced form of XBP-1/β-actin were determined by semi-quantitative PCR (n = 3-4). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

Effect of genistein on inflammatory response

The hepatic mRNA expressions regarding inflammatory genes were shown in **Fig. 8.** Because fat accumulates in the liver, there is sustained hepatic generation of pro-inflammatory cytokines from the Kupffer cells, leading to a vicious cycle of worsening severity of steatohepatitis. Thus, we measured expression of genes involved in inflammation. Consistent with increased inflammatory foci, MCD diet significantly induced the mRNA levels of genes related to inflammation. TNF-α; a key pathological factor that plays a role in almost all steps of NASH development, MCP-1; the key chemokine that regulates migration and infiltration of macrophages [77], TLR4; one of the key components of the receptor for lipopolysaccharide and IL-1β; key player in the regulation of inflammatory processes. Hepatic mRNA expressions for TNFα, MCP-1, TLR4 and IL-1β were up-regulated by MCD diet. However, Theses inflammatory genes expressions were significantly alleviated by genistein supplementation.

The hepatic adiponectin resistance has been suggested to be involved in steatohepatitis progression by changing PPARα activity and ROS accumulation [78]. Therefore, we determined whether genistein modulate AMPK pathway in MCD-mediated steatohepatitis model. Feeding an MCD diet significantly inhibited AMPK activation. Genistein supplementation restored the relative expression

levels of activated AMPK proteins (**Fig. 9A**). Furthermore, mRNA levels of PPARα, a downstream transcription factor of adiponectin signaling, were significantly decreased in mice fed an MCD diet (**Fig. 9B**). Although genistein supplementation tended to increase PPARα mRNA levels, the difference was not significantly different. As previously reported [79], adiponectin resistance was not associated with an altered adiponectin receptor expression in livers of MCD-fed mice (data not shown).

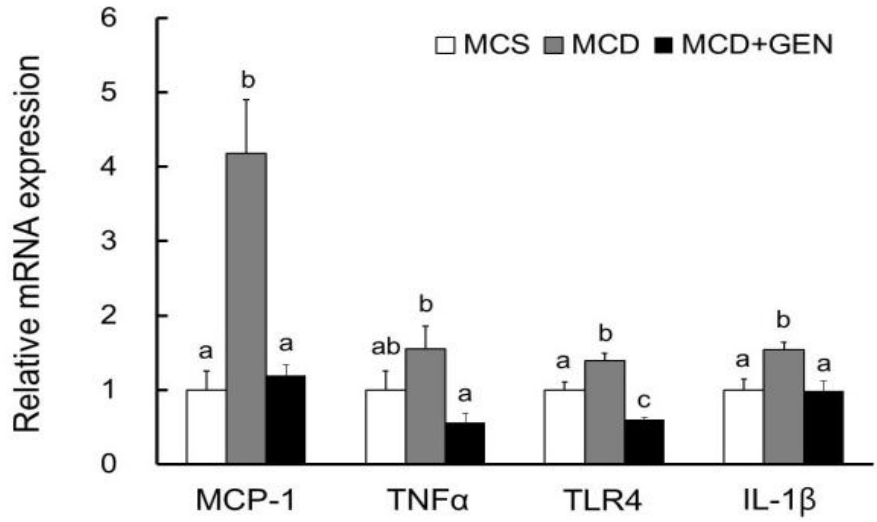
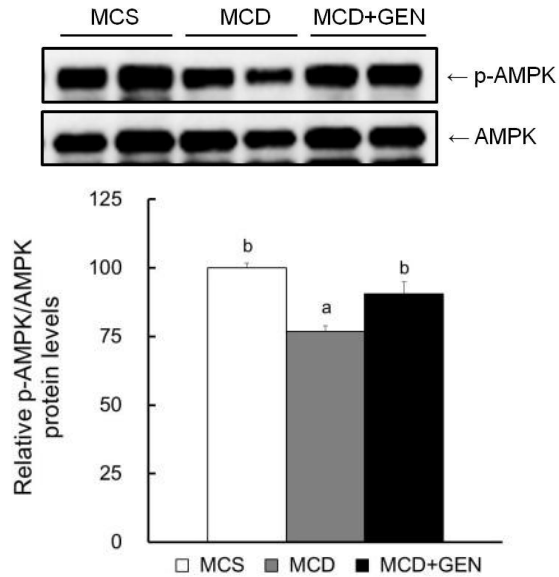


Figure 8. Effect of genistein on hepatic inflammation markers

Relative mRNA expression of genes involved in inflammation was determined by qPCR and was normalized to RPL19 (n = 3-4). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

(A)



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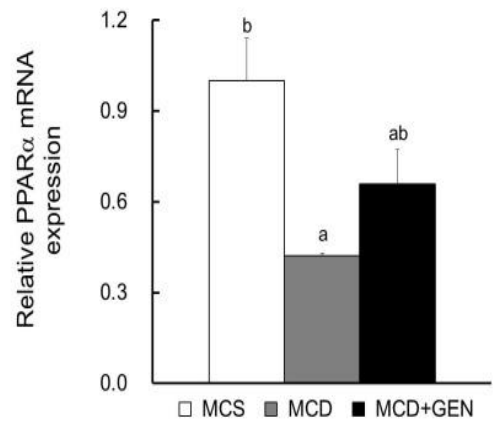

Figure 9. Effect of genistein on hepatic adiponectin resistance

(A) Relative p-AMPK/AMPK protein levels were determined by immunoblotting (n = 3). **(B)** Relative mRNA expression of PPARα/RPL19 was determined by qPCR (n = 3). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

Effect of genistein on liver fibrosis

To examine the effects of genistein supplementation on liver fibrosis, sections of liver were stained with Masson's trichrome. Mild perivenular fibrosis was observed in MCD-fed mice (**Fig. 10**). Genistein apparently reduced Masson's trichrome-stained fibrils induced by an MCD diet.

Parallel to the changes in histological fibrosis, the increased hepatic TGFβ, collagen, type I, alpha 1 (COL1A1) and tissue inhibitors of metalloproteinase 1 (TIMP1) mRNA levels in MCD diet-fed *db/db* mice were significantly. TGFβ triggers activation of HSC. TIMP1 plays a crucial role in the pathogenesis of hepatic fibrosis, it has been shown to be increased in liver fibrosis development. There was a tendency of mRNA levels involved in hepatic fibrosis to decrease in genistein treatment groups (**Fig. 11**).

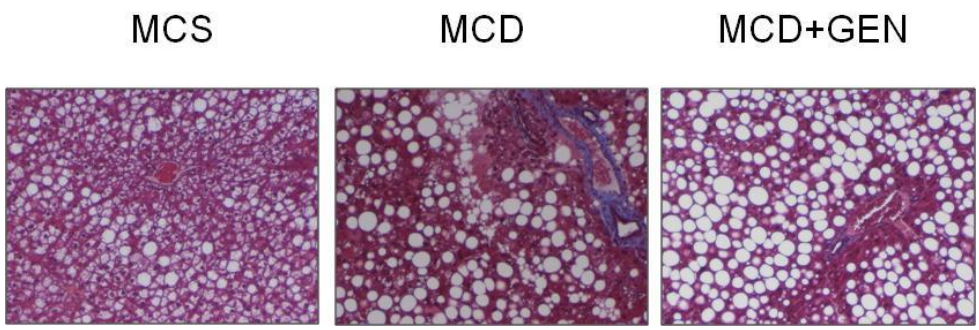

Figure 10. Representative Masson's trichrome staining of liver sections

The Masson's trichrome staining of liver tissue sections (n = 4) for collagen (stained blue) highlights perivenular (chicken wire) fibrosis, which is characteristic of fibrosis in NASH.

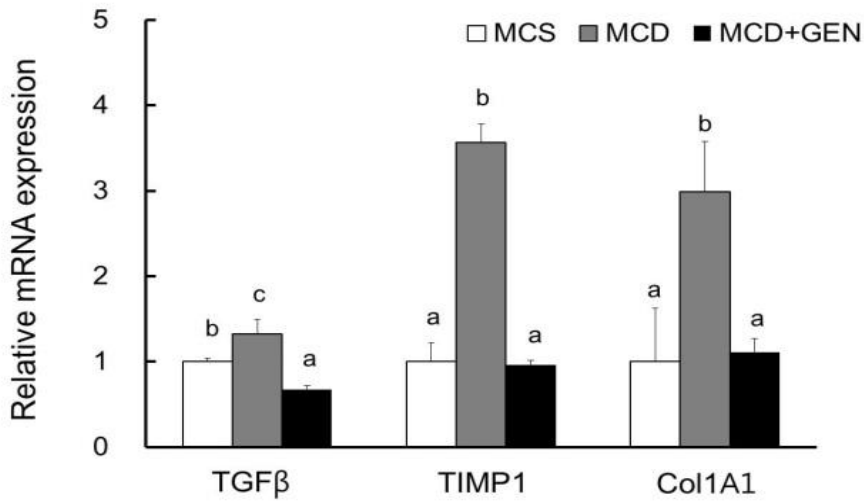

Figure 11. Effect of genistein on hepatic fibrosis markers

(A) Representative Masson's trichrome staining of liver tissue sections (n = 4). **(B)** Relative mRNA expression of genes involved in fibrosis was determined by qPCR and was normalized to RPL19 (n = 3-4). Each bar represents the means ± SEM and bars with different superscripts are significantly different at $P < 0.05$.

Discussion

It has been reported that genistein has been shown to attenuate NAFLD induced in obesity [64], type 2 diabetes animal models [68] and NASH using hyperlipidemic animal models [80]. However, there is no study regarding the effect of genistein on a NASH-related fibrosis. Moreover, there have been no studies of effect of genistein on dietary NASH model which has significant weight loss. Therefore, it is unclear to determine whether the effect of genistein against NASH is the secondary effect following the weight loss. Thus we aimed to clarify whether the anti-NASH effect of genistein should be accompanied with anti-obesity effect in *db/db* mice fed an MCD diet for 6 weeks. MCD diet-mediated NASH model in *db/db* mice is the useful to elucidate whether genistein could alleviate progression of steatohepatitis to liver fibrosis, which is not easily induced in dietary NASH models, although this animal model has its own limitations, including the lack of obesity and peripheral insulin resistance [81].

The ‘Two hit hypothesis’ has become a prevalently accepted framework for understanding the pathogenesis of NAFLD. In MCD model, the first hit is the impaired mitochondrial β-oxidation and the second hit is promotes oxidative stress and lipid peroxidation [25], which induce pro-inflammatory gene expression and

hepatic stellate cell activation that produce collagen and lead to liver fibrosis [10, 20, 82]. There are several reports suggested that oxidative stress involved in the development of NASH [19, 83, 84]. In the present study, the supplementation of genistein attenuated the oxidative stress in the liver of mice fed an MCD diet. Hepatic TBARS levels increased in *db/db* mice fed an MCD diet and genistein decreased hepatic TBARS levels. Additionally, genistein decreased mRNA level of HO-1 increased in *db/db* mice fed an MCD diet, suggesting that genistein supplementation alleviated oxidative stress. Likewise, anti-oxidants have been shown to prevent the development and progression of NASH in mice fed the MCD diet [85, 86].

In addition to oxidative stress, ER stress has been shown to be induced in an MCD diet-induced NASH model. A previous study showed that Hep3B cells exposed to TNFα activated ER stress [87]. Also, ER stress has been developed in the liver and adipose tissue of the patients with NAFLD or obesity [88, 89]. A recent study demonstrated that the MCD diet causes up-regulation of the UPR markers [46, 90]. Accordingly, we observed significant elevation in hepatic ER stress markers including BiP, CHOP, Bax and Xbp-1 in *db/db* mice fed MCD diet. However, the levels of expression of ER stress markers are alleviated by genistein.

Furthermore, hepatic adiponectin resistance may be involved in accelerating NASH development in *db/db* mice fed an MCD diet [78]. The

consequences of adiponectin signaling, mediated through the adiponectin receptors, include inhibition of fatty acid synthesis, enhancement of fatty acid oxidation and reducing of inflammatory processes. These outcomes are mediated by the dual effects of AMPK activation (by phosphorylation), and increased PPARα activity [79]. In the present study, MCD diet significantly inhibited phosphorylation of AMPK. Genistein supplementation restored the activation of AMPK proteins. Additionally, mRNA levels of PPARα were significantly decreased in MCD diet group. Although supplementation of genistein increased PPARα mRNA levels, it was not significant.

Inflammation is also accepted as an important mediator in the progression of NASH [91]. On a molecular level, macrophage infiltration in to the liver is mainly controlled by MCP-1. In the injured liver, MCP-1 in synthesized by activated stellate cells, hepatocytes and macrophages, thereby representing a principal and redundantly activated pathway of inflammation [92]. In the previous studies, TLR4 plays prominent roles in the turning of stages from steatosis to NASH [93]. Moreover, it is well known that TNFα and IL-1β are key players in the regulation of inflammatory responses. We observed that treatment of MCD diet-fed mice with genistein reduced mRNA levels of markers involved in inflammatory responses including MCP-1, TLR4, TNFα and IL-1β. These results suggest that the inhibitory effect of genistein on inflammasome activation, which has been shown

to be involved in hepatocyte pyroptosis, liver inflammation and fibrosis [94].

Activation of HSCs along with fibrogenesis reflects the clinical severity of NASH [95]. Although the underlying mechanisms remain unknown, a large body of evidence supports the role of oxidative stress in the onset of fibrosis. Excess ROS and lipid peroxidation products play a major mediatory role in induction of extracellular fibrillar matrix synthesis. A previous study reported that genistein had positive effects on liver fibrosis in rats administered CCl4 [96, 97] and in rats chronically administrated of alcohol [98]. Similarly, we also observed that genistein inhibited collagen accumulation and HSC activation. These results indicate that genistein supplementation attenuates liver fibrosis via the inhibition of collagen synthesis and the activation of the extracellular matrix degradation via the inhibition of TIMP expression in the liver.

In this study, MCD diet supplemented with genistein (500mg genistein/kg diet). The dose of genistein supplementation is based on a previous studies that reported mice fed AIN-93G diet supplemented with various dosages of genistein (125-1000mg/kg diet) for 24 weeks exhibited 0.39-3.36 μM/L levels plasma total genistein [99] and mice fed AIN-76A diet supplemented with genistein (750mg/kg diet) which has been reported to yield serum genistein levels in mice (~1-2 μM/L) similar to those observed in humans consuming a diet containing modest amounts of soy products (1-2 servings) [100].

In conclusion, it is clear that dietary supplementation of genistein alleviation liver inflammation and fibrosis in the MCD diet-induced NASH model. The mechanisms of genistein-mediated protection from MCD diet-induced liver fibrosis likely include inhibition of oxidative and ER stress, and pro-inflammatory cytokine production, modulation of impaired PPARα-mediated signaling and inhibition of collagen deposition. Especially, the results from our model show that at least in the MCD diet-mediated NASH model, the benefit of the genistein supplementation on liver inflammation and fibrosis may occur even in the absence of significant changes in triglyceride accumulation in the liver. Therefore, our finding provide strong support for the hypothesis that genistein prevents liver inflammation and fibrosis mediated by MCD diet in *db/db* mice.

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국문 초록

제니스테인의 보충섭취가 *db/db* 마우스에서 메티오닌-콜린 결핍 식이에 의해 유도된 비알코올성 지방간질환에 미치는 영향

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유나영

비알코올성 지방간질환은 간세포에 지방의 과도한 축적만 일어나는 단순지방증에서 지방간염, 섬유증 그리고 드물게 간경변증까지 발병되는 일련의 질환군을 의미한다. 제니스테인은 비만과 제 2 형 당뇨모델에서 유도된 비알코올성 지방간염을 완화시키는 것으로 선행연구를 통해 보고되었다. 하지만 간섬유화가 진행된 비알코올성 지방간염 수준에서의 연구는 미비한 실정이다. 또한 메티오닌-콜린 결핍 식이처럼 체중감소를 동반하는 모델에서는 전혀 연구가 되지 않았기에 항지방간염 효과가 체중저하에 따른 부가적인 작용인지 아니면 제니스테인의 직접적인 작용인지 명확하지 않다. 따라서 본 연구는 *db/db* 마우스에 메티오닌-콜린 결핍 식이를 공급하여 섬유화증을 동반한 지방간염모델을 설정하여 제니스테인의 섭취에 따른 개선효능을

규명하고자 하였다. 수컷 C57BL/KsJ-*db/db* 마우스를 정상식이군과 메티오닌-콜린 결핍 식이군 및 0.05% 제니스테인이 첨가된 메티오닌-콜린 결핍 식이군으로 나누어 6 주간 식이를 공급하였다. 메티오닌-콜린 결핍 식이군에서 유의적인 체중과 간조직 무게 감소가 일어났다. 메티오닌-콜린 결핍 식이는 혈청 포도당, 인슐린, 콜레스테롤, 알라닌 아미노 전이 효소 수준을 낮춰 주었다. 또한 산화스트레스, 소포체스트레스, 지방간증, 염증반응, 간성상세포활성 및 경섬유화증 등이 메티오닌-콜린 결핍식이 군에서 유도되었다. 제니스테인의 보충섭취는 혈청 인슐린 수준을 낮추어 주었으나, 제니스테인의 보충섭취로 인한 지방간증 저해효과는 관찰되지 않았다. 하지만 산화스트레스, 소포체스트레스 및 AMP-activated protein kinase 비활성이 제니스테인의 보충섭취로 인해 완화됨을 확인하였다. 제니스테인은 또한 염증반응 관련 사이토카인과 섬유증 관련 유전자의 발현수준을 감소시켜 주었다. 따라서 제니스테인은 지질과산화, 염증반응 및 경섬유화증으로의 진전을 효과적으로 억제하여 *db/db* 마우스에서 메티오닌 콜린 결핍식이로 유도된 지방간염을 개선시키는데 효과가 있을 것으로 사료된다.

주요어: *db/db* 마우스, 섬유증, 제니스테인, 메티오닌-콜린 결핍 식이, 비알코올성 지방간염

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