

Inhibitory effects of *Doenjang*, Korean traditional fermented soybean paste, on oxidative stress and inflammation in adipose tissue of mice fed a high-fat diet

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BACKGROUND/OBJECTIVES: *Doenjang*, Korean traditional fermented soybean paste has been reported to have an anti-obesity effect. Because adipose tissue is considered a major source of inflammatory signals, we investigated the protective effects of *Doenjang* and steamed soybean on oxidative stress and inflammation in adipose tissue of diet-induced obese mice.

MATERIALS/METHODS: Male C57BL/6J mice were fed a low fat diet (LF), a high-fat diet (HF), or a high-fat containing *Doenjang* diet (DJ) or a high-fat containing steamed soybean diet (SS) for 11 weeks.

RESULTS: Mice fed a DJ diet showed significantly lower body and adipose tissue weights than those in the HF group. Although no significant differences in adipocyte size and number were observed among the HF diet-fed groups, consumption of *Doenjang* alleviated the incidence of crown-like structures in adipose tissue. Consistently, we observed significantly reduced mRNA levels of oxidative stress markers (heme oxygenase-1 and p40^{phox}), pro-inflammatory adipokines (tumor necrosis factor alpha and macrophage chemoattractant protein-1), macrophage markers (CD68 and CD11c), and a fibrosis marker (transforming growth factor beta 1) by *Doenjang* consumption. Gene expression of anti-inflammatory adipokine, adiponectin was significantly induced in the DJ group and the SS group compared to the HF group. The anti-oxidative stress and anti-inflammatory effects observed in mice fed an SS diet were not as effective as those in mice fed a DJ diet, suggesting that the bioactive compounds produced during fermentation and aging may be involved in the observed health-beneficial effects of *Doenjang*.

CONCLUSIONS: *Doenjang* alleviated oxidative stress and restored the dysregulated expression of adipokine genes caused by excess adiposity. Therefore, *Doenjang* may ameliorate systemic inflammation and oxidative stress in obesity *via* inhibition of inflammatory signals of adipose tissue.

Nutrition Research and Practice 2015;9(3):235-241; doi:10.4162/nrp.2015.9.3.235; pISSN 1976-1457 eISSN 2005-6168

Keywords: Adipose tissue, *Doenjang*, inflammation, obese mice, oxidative stress

INTRODUCTION

The prevalence of obesity has shown a considerable increase and has become a serious global health problem in recent decades. Obesity, particularly excess visceral adiposity, is accompanied by a chronic low-grade inflammation, which contributes to the development of metabolic dysfunction, such as dyslipidemia, insulin resistance, non-alcoholic steatohepatitis, and cardiovascular diseases [1]. White adipose tissue (WAT) has traditionally been considered as a main energy reservoir in the body; however, it is now well established as an active endocrine organ responsible for metabolic homeostasis in the body through communication with a wide variety of organs [2].

In the development of obesity, excessive lipids are accumulated

in enlarged WAT, and then hypertrophic adipocytes trigger cellular stress including inflammation, oxidative stress and fibrosis within WAT [3]. In particular, the link between obesity and its pathophysiological effects is associated with macrophage accumulation in adipose tissue [3,4]. Monocyte chemoattractant protein 1 (MCP-1) is activated to recruit macrophages into adipose tissue. Necrotic dead adipocytes are surrounded by an aggregation of single or fused macrophages, forming a characteristic histological feature "crown-like structure (CLS)". In addition, phenotype of infiltrated macrophages is polarized from the M2 phenotype (alternatively activated macrophage) to M1 phenotype (classically activated macrophage), which highly secretes pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α), interleukin (IL)-6, and IL-1 β . TNF- α activates nuclear

This research was supported by the Globalization of Korean Foods R&D program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea (No. 913008-1).

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Received: December 19, 2014, Revised: February 3, 2015, Accepted: February 16, 2015

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factor kappa B (NF- κ B) signaling and c-Jun N-terminal kinases (JNK) signaling, which stimulate the release of pro-inflammatory cytokines [5]. In addition, TNF- α negatively regulates the expression of adiponectin, an anti-inflammatory and insulin sensitizing adipokine [6]. Tissue inflammation has been shown to be tightly associated with the accumulation of extracellular matrix components, which leads to the dysfunction of various organs, such as the liver, heart, and kidney [7]. Therefore, it is becoming increasingly evident that a localized inflammation within adipose tissue can lead to an overall systemic inflammation, which contributes to the development of obesity-linked complications.

Doenjang is a basic seasoning traditionally used in Korea. When made traditionally, *Doenjang* is manufactured from a cooked and crushed soybean block, *Meju*, which is then fermented with various microorganisms [8]. Previous studies have shown that fermented soybean foods contain higher levels of bioactive compounds [8,9], and exhibited stronger anti-oxidative [10] and anticancer activities [11,12] than unfermented ones. Most isoflavones in cooked and in unfermented soybean exist in the glucoside form. During the process of fermentation, cleavage of β -glycosyl bond of isoflavone glucoside by the rapid growth of microbes results in higher content of isoflavone aglycones, including genistein and daidzein. Recently, the functionality of various fermented soybean foods has been studied extensively in both rodents and humans. Dietary feeding of *Doenjang* led to markedly suppressed body weight gain, and serum oxidative stress and cytokine levels in high-fat-fed mice [10,13,14]. Daily consumption of *Doenjang* for 12 weeks leads to reduction in body weight and body fat mass of overweight adults [15]. In particular, genistein, the most abundant soy isoflavone [16], plays an important role in regulation of lipid metabolism, and inhibits development of high-fat diet-induced obesity and non-alcoholic fatty liver disease [17,18]. However, the anti-inflammatory and anti-oxidative stress effects of *Doenjang* in adipose tissue have not been investigated. Therefore, in the current study, we investigated whether *Doenjang* containing high isoflavone in aglycone forms inhibits obesity-associated inflammation in adipose tissue of mice fed a high-fat diet.

MATERIAL AND METHODS

Animals and diets

Male C57BL/6J mice at 4 weeks of age were purchased from Nara Biotech Co. (Korea). After an acclimation period of approximately 1 week of, mice were randomly divided into 4 groups and each group was fed the respective diets (Unifaith Inc., Korea) for 11 weeks; a low fat diet (LF, n = 12), a high-fat diet (HF: 45% fat and 1% cholesterol, n = 12), a high-fat containing 14.4% freeze-dried *Doenjang* diet (DJ, n = 11) and a high-fat containing 11.7% freeze-dried steamed soybean diet (SS, n = 12). The dose of *Doenjang* is based on a previous study, which showed that feeding 20% DJ for 8 weeks is effective in anti-obesity [14]. In an SS diet, 11.7% of steamed soybean was added to adjust the soy protein intake to the level of a DJ diet. Macronutrient content in DJ and SS diets was adjusted to those in an HF diet by addition of casein, soybean oil, corn starch, and fiber. Traditionally prepared *Doenjang* (aged for 6 months) and steamed soybean were obtained from the Institute of

Table 1. Experimental diet composition

| Ingredient (g) | LF | HF | DJ | SS |
|---|-------|-------|--------|--------|
| Casein | 200.0 | 200.0 | 152.9 | 152.9 |
| Corn starch | 376.7 | 151.0 | 131.95 | 132.71 |
| Maltodextrin | 100.0 | 100.0 | 100.0 | 100.0 |
| Sucrose | 172.8 | 200.0 | 200.0 | 200.0 |
| Lard | - | 188.5 | 188.5 | 188.5 |
| Soybean oil | 50.0 | 50.0 | 22.7 | 26.75 |
| Cholesterol | - | 10.0 | 10.0 | 10.0 |
| Cellulose | 50.0 | 50.0 | 36.05 | 27.46 |
| AIN 93 mineral mix | 35.0 | 35.0 | 35.0 | 35.0 |
| AIN 93 vitamin mix | 10.0 | 10.0 | 10.0 | 10.0 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 | 2.5 |
| L-cystine | 3.0 | 3.0 | 3.0 | 3.0 |
| Doenjang, freeze-dried ¹⁾ | - | - | 150 | - |
| Steamed soybean, freeze-dried ²⁾ | - | - | - | 118 |
| Total | 1000 | 1000 | 1042 | 1006 |
| Protein (kcal%) | 20.8 | 16.8 | 16.8 | 16.8 |
| Carbohydrate (kcal%) | 67.5 | 38.0 | 38.0 | 38.0 |
| Fat (kcal%) | 11.7 | 45.2 | 45.2 | 45.2 |
| Energy (Kcal/g) | 3.85 | 4.75 | 4.56 | 4.72 |

LF: low fat, HF: high-fat, DJ: Doenjang, SS: Steamed soybean.

¹⁾ Containing 12.7% carbohydrate, 18.2% fat, 31.4% protein and 9.3% fiber.

²⁾ Containing 15.5% carbohydrate, 19.7% fat, 39.9% protein and 19.1% fiber.

Sunchang Fermented Soybean Products (Korea), and were freeze-dried, powdered and stored at -20°C. Freeze-dried *Doenjang* and steamed soybean were analyzed for nutritional composition and isoflavone content by Korea Food Research Institute and Research Institute for Food Safety at Optipharm Co. (Korea). The composition of diets is shown in Table 1. Animals were maintained in a temperature (21 \pm 2°C) and humidity (50 \pm 20%)-controlled environment with a 12 h dark-light cycle, and had *ad libitum* access to their respective food and water throughout the study. The experimental protocol was approved by the Chungbuk National University Institutional Animal Care and Use Committee (CBNUA-636-13-01). After overnight fasting, mice were sacrificed by CO₂ asphyxiation. Tissues were removed, quickly frozen in liquid nitrogen and stored at -80°C until analysis. Blood was collected by cardiac puncture and serum leptin levels were analyzed using an ELISA kit (#EZML-82K; EMD Millipore, USA).

Adipose tissue histologic examination

Formalin-fixed adipose tissue was processed into 4- μ m-thick paraffin sections and stained with hematoxylin and eosin (H&E) for histological evaluation. The morphology was observed under an Olympus BX50 microscope using a DP-72 digital camera (Olympus, Japan) and the image was captured using Image-Pro Plus ver. 4.5 program (Media Cybernetics Inc., USA). The size and number of adipocytes per each field of view were measured at 200 \times magnification.

Total RNA extraction and quantitative PCR (qPCR) analyses

The total RNA of mouse epididymal adipose tissue was isolated using the RNAiso Plus (Takara Bio Inc., Japan) and cDNA

was synthesized with Superscript[®]II Reverse Transcriptase (Invitrogen, USA). 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) according to the supplier's protocol. 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. For amplification of cDNA, primers for TNF- α (forward 5'-GGCTACA GGCTTGRCACTCGA-3', reverse 5'-CACGCTCTTCTGTCTACTGAA-3'), MCP-1 (forward 5'-CCAGCACCAGCACCAGCCAA-3', reverse 5'-TGGGGCGTAACTGCATCTGGC-3'), CD68 (forward 5'-GCACAG CCAGCCCTACGA-3', reverse 5'-GAGCTGGTGTGAAGTGTGACATTT-3'), CD11c (forward 5'-CTGGATAGCCTTTCTTCTGCTG-3', reverse 5'-GCACACTGTGCCGAATC-3'), transforming growth factor beta 1 (TGF- β 1; forward 5'-CACCGAGAGCCCTGGATA-3', reverse 5'-TGTACAGTCCGCACACA-3'), pro-collagen, type I, alpha 1 (COL1A1; forward 5'-GCTCTCTTAGGGCCACT-3', reverse 5'-CCAGTCTCACCATTGGGG-3'), heme oxygenase-1 (HO-1; forward 5'-CCTCACTGGCAGGAAATCATC-3', reverse 5'-CCTCGTGGAGACG CTTTACATA-3'), adiponectin (forward 5'-CCGTCTCTTACCTA CGAC-3', reverse 5'-TCCCCATCCCCATACAC-3') and RPL19 (forward 5'-TCAGGCTACAGAAGAGGCTTGC-3', reverse 5'-ATCAGCCCATCC TTGATCAGC-3') were used.

Protein extraction and immunoblotting

The epididymal tissues (~100 mg) were homogenized in 300 μ L of 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 Na₂VO₄, 1 mM DTT, 1% NP-40, 10% glycerol, 0.2 mM PMSF and Protease inhibitor cocktail (Sigma, USA)] using the Tissue Lyser system (Qiagen, USA) with 5 mm sterile stainless steel beads. After centrifugation for 30 min at 10,000 \times g at 4°C, the protein content of the supernatant was measured using a protein assay kit (Bio-Rad, USA). Equal amounts of protein were loaded into the lanes of an SDS-PAGE gel, separated and then transferred to a PVDF membrane using a semi-dry electrotransferring unit (Bio-Rad). After blocking with 5% nonfat milk or bovine serum albumin in TTBS, membranes were probed with specific antibodies diluted in TTBS with 5% nonfat milk or bovine serum albumin as follows: anti-catalase (ab-16731, Abcam, USA), anti-HO-1 (sc-10789, Santa Cruz Biotechnology, USA), anti-p-JNK (s-9251, Cell signaling, USA), anti-70-kDa heat shock cognate protein (HSC70; Santa Cruz Biotechnology) or anti-beta-actin (A5441, Sigma, USA). The membranes were then incubated with an IgG-peroxidase-conjugated secondary antibody for chemiluminescent detection. The band intensities were quantified using Quantity One software (Bio-Rad).

Statistical analysis

SPSS software (ver. 21.0, SPSS Inc., USA) was used for data analysis. For all experiments, one-way ANOVA followed by Duncan's multiple range test was employed to assess the statistical significance. Data were expressed as the mean \pm SEM and differences were considered statistically significant at $P < 0.05$. Correlations between two variables were determined by Pearson's correlation coefficient.

RESULTS

Effects of Doenjang on body and adipose tissue weights in mice fed a high-fat diet

At the end of the experiment, *Doenjang* consumption had significantly reduced the final body weight of mice fed an HF diet (Table 2). The body weight of mice was significantly lower in the DJ group than in the HF group from week 1 (Fig. 1). Food intake was not significantly different among mice fed an HF diet (data not shown). Consistently, *Doenjang* consumption led to significantly reduced epididymal, retroperitoneal, and perirenal fat weights in mice fed an HF diet (Table 2). Steamed soybean consumption did not result in significant change in both body and adipose tissue weights. High-fat feeding led to significantly increased serum leptin levels, which were significantly reduced in mice fed a DJ diet.

Effects of Doenjang on adipose tissue morphology of mice fed a high-fat diet

Microscopic examination of H&E staining of adipose tissue showed adipocyte hypertrophy and inflammation in mice fed an HF diet (Fig. 2A). We observed significantly increased size and decreased number of adipocytes in mice fed an HF diet compared to mice fed an LF diet (Fig. 2B, C). Consumption of

Table 2. Body weight, adipose tissue weight and serum leptin levels of mice fed a high-fat diet containing *Doenjang*

| | LF | HF | DJ | SS |
|---------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| Body weight (g) | | | | |
| Initial weight | 18.7 \pm 0.2 | 18.7 \pm 0.3 | 18.7 \pm 0.2 | 18.7 \pm 0.2 |
| Final weight | 29.3 \pm 0.7 ^b | 38.0 \pm 1.0 ^a | 31.8 \pm 0.8 ^b | 38.8 \pm 0.7 ^a |
| Adipose tissue weight (g) | | | | |
| Epididymal | 0.87 \pm 0.08 ^c | 2.04 \pm 0.07 ^a | 1.65 \pm 0.12 ^b | 2.14 \pm 0.08 ^a |
| Retroperitoneal | 0.21 \pm 0.03 ^c | 0.56 \pm 0.04 ^a | 0.43 \pm 0.04 ^b | 0.55 \pm 0.03 ^a |
| Perirenal | 0.09 \pm 0.01 ^c | 0.20 \pm 0.01 ^a | 0.14 \pm 0.01 ^b | 0.19 \pm 0.01 ^a |
| Serum leptin (ng/mL) | 4.5 \pm 0.7 ^c | 13.0 \pm 0.6 ^a | 9.7 \pm 0.7 ^b | 14.0 \pm 0.6 ^a |

LF: low fat, HF: high-fat, DJ: Doenjang, SS: Steamed soybean. Results are given as the mean \pm SEM (n=11-12 for body and adipose tissue weights; n=10-11 for serum leptin levels). Means in the same row that do not share the same superscript are significantly different by ANOVA ($P < 0.05$).

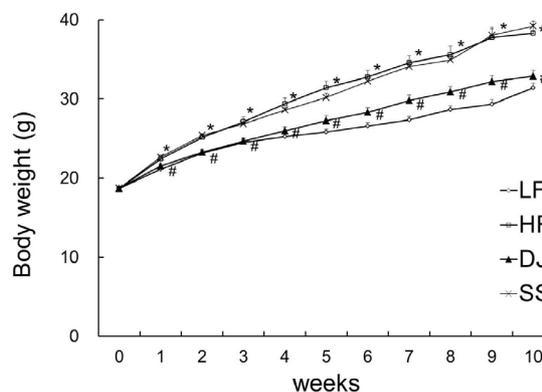


Fig. 1. Body weight change of mice fed a high-fat diet (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). Data are presented as the mean \pm SEM (n=10-11). * $P < 0.05$ vs. the low fat diet (LF) group, # $P < 0.05$ vs. the HF group by ANOVA.

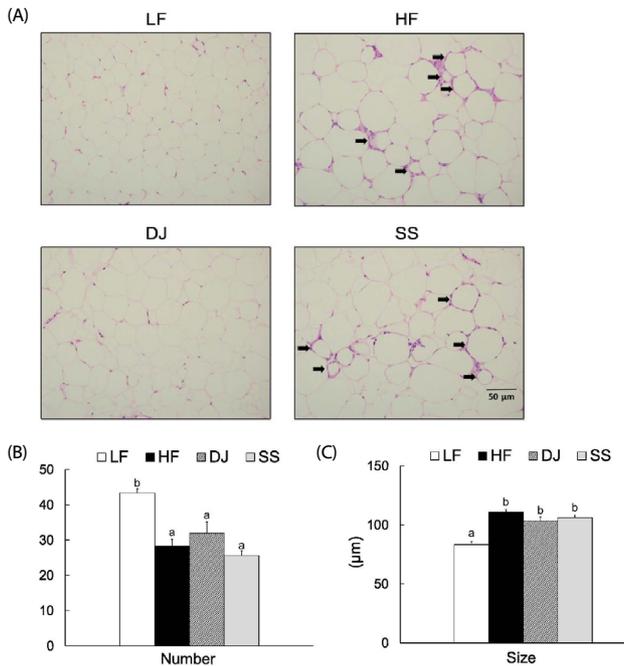


Fig. 2. Adipocyte morphology of mice fed a high-fat diet (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). (A) Representative H&E staining of adipose tissue sections (magnification 200 \times). Scale bar represents 50 μ m. Crown-like structures are indicated with the black arrow. (B) Number of adipocytes per field of observation. (C) Size of adipocytes. Each bar represents the mean \pm SEM (n=5) and bars that do not share the same superscript are significantly different by ANOVA ($P < 0,05$).

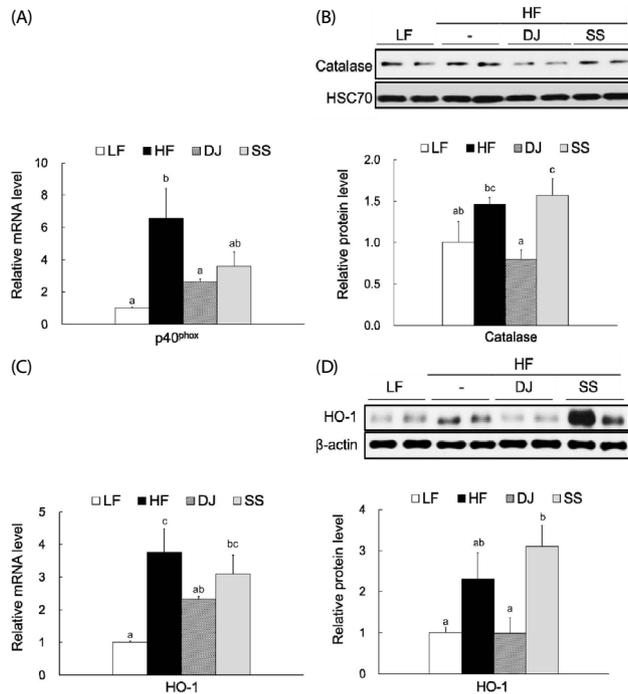


Fig. 3. Relative expression of oxidative stress markers in adipose tissue of mice fed a high-fat diet (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). (A) Relative mRNA expression of p40^{phox}/RPL19 was determined by qPCR (n=3-4). (B) Relative catalase/HSC70 protein levels were determined by immunoblotting (n=4). (C) Relative mRNA expression of HO-1/RPL19 was determined by qPCR (n=3-4). (D) Relative HO-1/ β -actin protein levels were determined by immunoblotting (n=4). Each bar represents the mean \pm SEM and bars that do not share the same superscript are significantly different by ANOVA ($P < 0,05$).

Doenjang and steamed soybean had no effect on adipocyte hypertrophy. CLSs, which were mainly observed in inflammatory adipose tissue, were observed less in the adipose tissue of the DJ group and the LF group compared to the HF group and the SS group (Fig. 2A).

Effects of *Doenjang* on oxidative stress in adipose tissue of mice fed a high-fat diet

Doenjang has been shown to have anti-oxidant activity via regulation of enzyme levels involved in cellular oxidative stress. We observed that mRNA levels of p40^{phox}, a subunit of NADPH oxidase, involved in superoxide generation [19], were significantly increased in mice fed an HF diet. Consumption of *Doenjang*, but not steamed soybean, resulted in significantly reduced mRNA levels of p40^{phox} (Fig. 3A). In addition, significantly lower protein levels of catalase were observed in the DJ group than in the HF group and the SS group (Fig. 3B). The mRNA levels of heme oxygenase (HO-1) were significantly induced in mice fed an HF diet, which were reduced by *Doenjang* consumption (Fig. 3C). Serum leptin levels showed significant association with mRNA levels of p40^{phox} ($r = 0,757$, $P = 0,004$) and HO-1 ($r = 0,906$, $P < 0,001$). Lower HO-1 protein levels were observed in the DJ group than in the HF group, however, the difference was not significant (Fig. 3D).

Effects of *Doenjang* on adipose tissue inflammation of mice fed a high-fat diet

Because we observed that consumption of *Doenjang* reduced CLS formation in adipose tissue of HF diet-fed mice, we assessed expression of pro-inflammatory and anti-inflammatory genes in adipose tissue. The mRNA levels of pro-inflammatory genes were significantly increased in the HF group compared to the LF group (Fig. 4A). Consistent with CLS formation, *Doenjang*

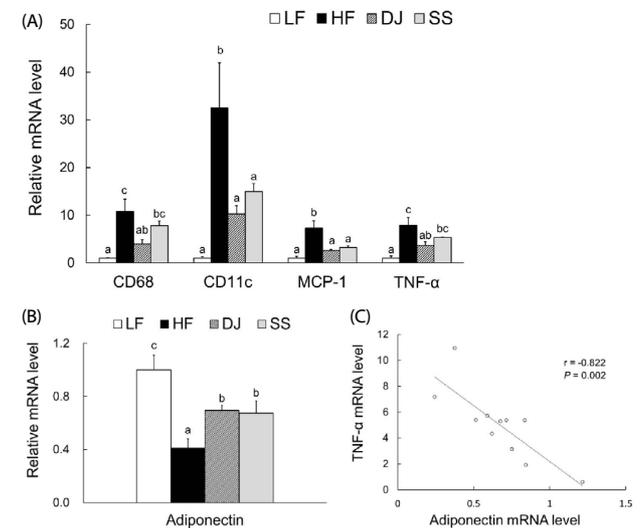


Fig. 4. Relative expression of inflammation markers in adipose tissue of mice fed a high-fat diet (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). Relative mRNA expression of genes involved in (A) pro-inflammation and (B) anti-inflammatory adipokine was determined by qPCR and was normalized to RPL19. Each bar represents the mean \pm SEM (n=3-4) and bars that do not share the same superscript are significantly different by ANOVA ($P < 0,05$). (C) Pearson's correlation between relative adiponectin mRNA expression and relative TNF- α mRNA expression.

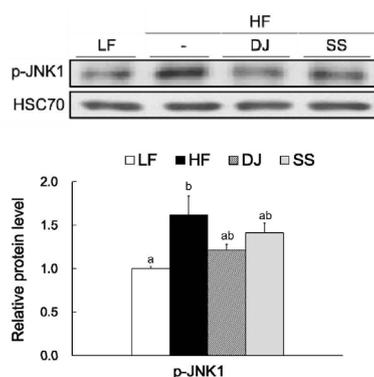


Fig. 5. Activation of inflammatory signaling in adipose tissue of mice fed a high-fat (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). Relative p-JNK1/HSC70 protein levels were determined by immunoblotting. Each bar represents the mean \pm SEM ($n=4$) and bars that do not share the same superscript are significantly different by ANOVA ($P<0.05$).

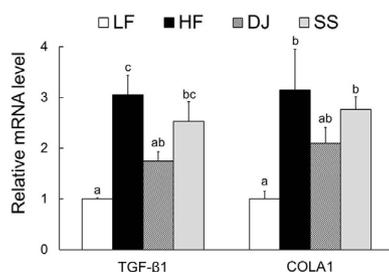


Fig. 6. Relative mRNA expression of fibrosis marker genes in adipose tissue of mice fed a high-fat (HF) and HF containing *Doenjang* (DJ) or steamed soybean (SS). Each bar represents the mean \pm SEM ($n=3-4$) and bars that do not share the same superscript are significantly different by ANOVA ($P<0.05$).

consumption led to significantly reduced mRNA levels of TNF- α , MCP-1, CD68, a total macrophage marker [20] and CD11c, a surface marker of M1 type [21] in obese mice. The gene expression of adiponectin was significantly decreased in diet-induced obesity, which was significantly increased in both the DJ and SS groups (Fig. 4B). In addition, a strong negative correlation was observed between TNF- α and adiponectin mRNA levels (Fig. 4C). Along with genes involved in oxidative stress, mRNA expression of genes involved in inflammation showed significant correlation with serum leptin levels (CD68: $r=0.840$, $P=0.001$; CD11c: $r=0.771$, $P=0.003$; MCP-1: $r=0.784$, $P=0.003$ and TNF- α : $r=0.881$, $P<0.001$).

Because TNF- α induces pro-inflammatory cytokine secretion via activation of JNK signaling [22], we investigated whether *Doenjang* consumption modulates JNK activation in adipose tissue of mice fed an HF diet. Significantly increased phosphorylated JNK-1 levels were observed in the HF group compared to the LF group (Fig. 5). *Doenjang* consumption tended to lead to down-regulation of p-JNK1 level in adipose tissue of mice fed an HF diet.

Effects of *Doenjang* on adipose tissue fibrosis of mice fed a high-fat diet

To investigate whether increased inflammation is involved in the excess synthesis of fibrillar collagen in adipose tissue, we measured mRNA levels of genes involved in fibrosis develop-

ment (Fig. 6). High-fat feeding led to significantly increased mRNA levels of TGF- β 1 and COL1A1. Significantly lower mRNA levels of TGF- β 1, but not COL1A1, were observed in the DJ group compared with the HF group. No inhibitory effects were observed in mice fed an SS diet.

DISCUSSION

Loss of physiologic homeostasis caused by chronic inflammation has been linked to development of various diseases, including obesity, diabetes, cardiovascular disease, and cancer. It has been suggested that numerous chemokines and cytokines secreted from adipose tissue are the main contributors of systemic inflammation in obesity. Under conditions of obesity, different adipose tissue cells, including adipocytes, macrophages and stromal fraction vascular cells lead to production and secretion of adipokines [3]. Anti-obesity effects of *Doenjang* have been reported in previous studies [14,23], however, this study particularly evaluated the protective effects of *Doenjang* on oxidative stress, inflammation and fibrosis in adipose tissue using a high-fat diet-induced obese mice model.

Increased oxidative stress in adipose tissue is an early agitator of metabolic syndrome. Since macrophages are known to produce reactive oxygen species (ROS), it is possible that infiltrated macrophages have important roles in elevated oxidative stress in obese adipose tissue [24]. ROS can be generated by various intracellular enzymes. NADPH oxidase, a family of enzymes that transfer electrons from NADPH to oxygen, produces superoxide radical and H₂O₂ [19]. Expression of NADPH oxidase subunits was induced in adipose tissue of rodents and obese human subjects [24-26]. In the current study, *Doenjang* protected against oxidative stress via inhibition of ROS production and enhancement of ROS degradation, which was confirmed by lower mRNA levels of HO-1, a microsomal enzyme induced in response to a variety of stimuli such as heavy metals, oxidative stress, and cytokines [27].

Elevated oxidative stress has been regarded as a potential contributing factor in the accumulation of immune cells in adipose tissue. Therefore, reducing oxidative stress and inflammation in adipose tissue will positively influence the risk of metabolic syndrome [24]. It is of note that significantly reduced TNF- α mRNA levels were observed in the DJ group compared to the HF group. TNF- α is mainly overproduced by macrophages and contributes to dysregulated production or secretion of adipokines in adipose tissue [3]. In addition, the mRNA expressions of MCP-1, CD68, and CD11c were reduced in the DJ group compared to the HF group. This means that *Doenjang* suppresses secretion of pro-inflammatory cytokines and inflammatory signaling from M1 macrophage through prevention of macrophage infiltration and phenotypic switch of macrophage polarization in adipose tissue during obesity. We also observed that serum leptin levels were significantly reduced by consumption of *Doenjang*. Leptin, which has a helical cytokine-like structure, was shown to play a regulatory role in inflammation and oxidative stress in various tissues and cells [28-30]. On the other hand, adiponectin, an anti-inflammatory cytokine, showed protective actions on the development of various obesity-linked diseases and it is negatively regulated by TNF- α in adipose tissue

[31]. As expected, we observed that the mRNA expression of adiponectin was significantly increased in the DJ group compared to the HF group. These results suggest that *Doenjang* may alleviate obesity-linked metabolic disorders by regulation of altered adipokine production.

Inflammation and hypoxia are tightly associated with an excess synthesis of fibrillar collagens and a failure of degradation of these proteins in adipose tissue [7]. Abnormal collagen deposition can exhibit considerable phenotype modulation with deleterious effects on tissue homeostasis and function [32]. TGF- β is an important regulator of fibrosis by enhancement of alpha smooth muscle actin (α -SMA) and COL1A1 [33]. We observed that consumption of *Doenjang* led to reduced mRNA levels of fibrosis markers, suggesting the inhibitory effect of *Doenjang* on adipose tissue fibrosis in obesity. Genistein has been shown to exert anti-fibrotic effects by suppression of hepatic stellate cell proliferation and α -SMA expression in response to platelet-derived growth factor [34].

In the current study, we observed the resolution of inflammation and fibrosis without changes in number and size of adipocytes by *Doenjang*. Similarly, a previous study reported that dipeptidyl peptidase-4, which degrades numerous peptides and chemokines, reduced expression of inflammation markers without affecting number and size of adipocytes [35]. It is reported that increased extracellular matrix components may interfere with adipose mass expansion in metabolically dysfunctional adipose tissue [1]. Accordingly, enhanced fibrosis with limited adipocyte hypertrophy was observed in obese humans [36]. In addition, macrophages are likely to outnumber adipocytes in adipose tissue [32], suggesting that reduced number of macrophages may contribute to lower adipose tissue weights in the DJ group compared to the HF group. Therefore, at least in the current study, *Doenjang* may exert its anti-obesity effects by inhibiting further crosstalk between adipocytes and macrophages, which comes after adipocyte hypertrophy. These qualitative changes in enlarged adipocytes by *Doenjang* may contribute to the transition of adipocytes from a metabolically dysfunctional phenotype to a metabolically functional phenotype.

In the current study, we included the SS group to investigate whether the fermentation and aging process of steamed soybean affect bioactive compounds involved in health beneficial effects of soybean and observed the stronger anti-obesity effect of *Doenjang* compared to steamed soybean in a diet-induced obese model. In addition, this is the first study to report that *Doenjang* exhibited anti-oxidative and anti-inflammation effects in adipose tissue of obese mice. Total content of isoflavone glucoside forms in a DJ diet and an SS diet was 5.76 and 336.96 mg/kg diet, respectively, and total content of isoflavone aglycone form in a DJ diet and an SS diet were 152.64 and 15.21 mg/kg diet, respectively. In particular, the content of both genistein and daidzein in a DJ diet was about 10-times higher than those in an SS diet. Increasing evidence shows that dietary isoflavones have antioxidant properties [37]. Genistein in particular has been shown to prevent the production of cytokines and ROS [17]. In addition, previous studies reported that soy bioactive peptides and protein fractions play an important role in protecting against chronic diseases [38]. Transformation of precursor proteins in soybean into bioactive peptides by microorganism

in fermented foods has been reported [39,40]. Collectively, these findings have led to the notion that isoflavone and soy peptides contribute to the anti-oxidative and anti-inflammatory effects of *Doenjang*.

In conclusion, dietary consumption of *Doenjang* led to amelioration of adipose tissue inflammation, oxidative stress, and fibrosis in a high-fat-induced obesity model. By contrast, steamed soybean did not exert these beneficial effects in adipose tissue of obese mice. Considering the potential to restore altered inflammatory mechanism, oxidative stress and fibrosis in adipose tissue, *Doenjang* may reduce the risk of metabolic syndrome development in obese subjects. Further research is warranted to investigate the bioactivity of *Doenjang* in prevention of chronic inflammatory diseases, including cardiovascular disease, diabetes, and autoimmune diseases.

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