Anti-obesity and Anti-inflammation Effects of *Cheonggukjang* in C57Bl/6 mice with High Fat Diet Induced Obesity

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The purpose of this *study* was to *investigate* the *anti-obesity and anti-inflammation effect* of the *cheonggukjang* (a soybean paste fermented for only a few days) in diet induced obesity mice. Weight gain was significantly decreased in the mice fed *cheonggukjang* compared High Fat Diets (HFD). The HFD plus *cheonggukjang* (CGJ) were also effective in improving the lipid metabolism. The levels of plasma triglyceride, cholesterol, ALT, AST, leptin, glucose, and insulin were significantly lower in CGJ than HFD group ($p<0.05$). The adiponectin level of CGJ group was significantly increased compared to the HFD group ($p<0.05$). In the CGJ group, the mRNA expression of adipogenic genes in the liver and adipose tissues, which are transcription factors crucial for adipogenesis, were significantly suppressed ($p<0.05$). The number of CD11b+F4/80+ T cells, Gr-1intCD11bhigh cells, and Gr-1intCD11bhigh cells were significantly higher in HFD group than CGJ group ($p<0.05$). The size of adipocyte was significantly reduced in CGJ group compared to HFD group. In addition, the contents of liver lipid droplets were significantly downregulated in the CGJ mice than HFD mice ($p<0.05$). Collectively, these data suggest the novel function of *cheonggukjang* in modulating adipogenesis through an immune function-alteration involving downregulation of adipogenic transcription factors and macrophage activation.

Key words : *Cheonggukjang*, DIO mice, inflammation, obesity, soybean

Introduction

The *cheonggukjang* is a traditional and famous fermented soybean food product in Korea, which is also alternatively called Natto, Tempeh and Douchi in other Asian countries. *Cheonggukjang* has traditionally been made with whole cooked soybean by fermented with *Bacillus subtilis* for 48 hr [42]. *Bacillus subtilis*, one of the major microorganisms, can be found in *cheonggukjang*. *Bacillus subtilis* manufactures strong proteolytic enzymes, and within the short fermentation period, soybean proteins are partially hydrolyzed into peptides [22]. Soybean peptide can inhibit the activity of angiotensin-converting enzyme, while preventing the contraction of peripheral vessels. It can also have a considerable effect on patients with cardiovascular disease [13]. During fermentation of *cheonggukjang*, through the activity of microbial β-glucosidase enzymes [4-6, 21], isoflavones are converted from glycosides into the corresponding aglycones and most proteins are degraded into small peptides and amino acids [29, 41]. Several studies have illustrated the valuable use of isoflavones which include prevention of mammary cancer [10], reduced risk of cardiovascular diseases [45], improvement of bone health and menopause symptoms [33, 17], antimutagenic effects [31, 32], and antidiabetic effects [24]. In addition to these, a number of studies in animals and humans suggest that consumption of soy protein have favorable effects on obesity and lipid metabolism. The anti-obesity effects of dietary soy protein were also reported in animal model such as genetically obese Wistar fatty rats which was evidenced by reduction of body weight, plasma and liver triacylglycerol concentrations, and lipogenic gene expression in livers [16]. Obesity, which is generally defined as an energy-rich condition, is characterized by the activation of an inflammatory process in metabolically active sites including adipose tissue, liver and immune cells [18]. Obesity is a complex, multifactorial, chronic disease involving environmental (social and cultural), genetic, physiologic, metabolic, behavioral, and psychological components [1]. Obesity is defined as a condition of excess body fat, and associated with a large number of life threatening disorders, such as cardiovascular, metabolic, and other non-communicable diseases [28]. Obesity is regarded as a major risk factor

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for non-alcoholic fatty liver disease (NAFLD). Dysfunction of hepatic immune cells was found to be involved in obesity-related hepatic diseases which were confirmed in hepatic steatosis animal model [36]. Macrophage surface marker F4/80 and CD11b, which are expressed on monocytes and neutrophils, are involved in inflammation associated with macrophage infiltration [46]. Neutrophils are professional phagocytes which are known to mainly function in recognition and destruction of pathogenic organisms. Also, they are most distinguished leukocyte in acute inflammatory reactions and thus accelerate host tissue injury in a number of inflammatory condition [30]. Accumulation of immature myeloid cells may be an important component in deteriorating obesity-triggered inflammation response of the hepatic tissue, which in turn worsens metabolic morbidity including steatohepatitis [7].

To investigate the effects of the *cheonggukjang* (CGJ) against obesity and inflammation, high-fat diet has been fed to C57BL/6 mice for 13 weeks to induce obesity. After the 13 weeks, along with the high-fat diet group, the obese mouse has been fed with the *cheonggukjang*. To study the effect and the level of repression, the food intake amount, change in weight gain, and the biochemical parameters of plasma have been measured. To understand the mechanisms how *cheonggukjang* can reduce lipid accumulation. mRNA expression levels of obesity-related genes along with numbers of macrophages and immature myeloid precursor cells were examined. The following have been measured through hematoxylin and eosin (H&E) staining: adipose size of adipose tissue, the level of infiltration in macrophage, spread and area of adipocyte.

The objective of this study is to determine the underlying mechanisms by which *cheonggukjang* inhibits adipogenesis and inflammation. To achieve this goal, C57BL/6J mice were fed with three different diets containing ND diet, HFD diet and HFD with *cheonggukjang*. Here, we report that inclusion of *cheonggukjang* in HFD were effectively modulates adipogenesis through an immune function involving downregulation of adipogenic transcription factors and macrophage activation.

Materials and Methods

Cheonggkukjang samples

Soybean was soaked for over 12 hr followed by cooking for 4 hours. It was cooled down to 50℃ before inoculation. Natural inoculation method using rice straws where *Bacillus subtilis* harbors was used. Rice straws were placed on the cooked soybean layer by layer and left at room for 2~3 days for fermentation where temperature and humidity were approximately 50℃ and 70%, respectively. Fermentation was terminated once sticky materials appeared on the surface of the cooked soybean. Methanol extracts of CGJ were prepared for cellular experiments and freeze-dried CGJ was prepared for the animal study.

Animals

Male C57BL/6J mice at 5 weeks (16-18 g) of age were purchased from the Central Lab. Animal Inc (Seoul, Korea). The mice were housed in cages of which temperature and humidity were kept at 22℃ and 45±5%, respectively. The light was controlled 12/12 hr (light/dark) cycle. Mice was fed a commercial chow diet and had free access to water for 1 week for adaptation. Animal study protocols were approved by the Committee for Animal Care at Pusan National University (PNU-2009-0010).

Diets

Low fat diet (D12450B) and high fat diets (D12451) used in this experiment were purchased from Research Diets Inc (New Brunswick, New Jersey, USA) [50]. Calorie from fat in the normal diet or high fat diet were accounted for 10 and 44.9% to their respective diet. CGJ freeze-dried and then added to the high fat diet to be 10%(w/w).

Diet induced obesity (DIO) experiment

After adaptation for 1 week, mice were divided into 3 groups. Normal diet group (ND, n=10) fed low fat diet (D12450B) for 26 weeks which provides 10% calorie from the fat. High fat diet group (HFD, n=10) fed high fat diet (45% calorie from fat, D12451) for 26 weeks. To see the anti-obesity effect of CGJ, CGJ diet group (CGJ, n=10) were fed high fat diet for 13 weeks to induce obesity (diet induced obesity) and then were fed high fat diet containing CGJ (10%, w/w) for another 13 weeks. Total duration for the experiment for the CGJ group was 26 weeks.

Preparation of plasma

After 13 and 26 weeks on the experimental diets, the mice were sacrificed with ethyl ether. Blood samples were collected into heparin treated tubes. Plasma was separated by centrifugation at 3,000 rpm for 15 min (VS-15CFU re-

frigerated centrifuge, Vision, Gyeonggi, Republic of Korea) and stored at -70℃ until further use.

Total cholesterol, triglyceride and glucose analysis

Concentration for total cholesterol kit (AM202K, Asan Pham, Seoul, Korea) triglyceride (AM1575K, Asan Pham, Seoul, Korea) and glucose (077K9806, Asan Pham, Seoul, Korea) of in the plasma were determined using commercially available kits.

AST and ALT

AST (aspartate aminotransferase, AM101-1, Asan Pham, Seoul, Korea) and ALT (alanine aminotransferase, AM101-2, Asan Pham, Seoul, Korea) levels of plasma were determined using commercially available kits.

Leptin, adiponectin, and insulin analysis

Determination of leptin, adiponectin, and insulin level in the plasma were performed with sandwich enzyme-linked immunosorbent assay (ELISA). For the leptin analysis, anti-mouse leptin, recombinant mouse leptin, and biotinylateed anti-mouse leptin antibodies were purchased from R&D Systems (MOB00, MN, USA) [11]. For adiponectin analysis, kit (MRP300) was purchased from R&D Systems (MN, USA) [44]. Insulin was detected by ELISA using commercially available kit (EZRMI-13K) from LINCO research (Missouri, USA) [23].

Assay for reverse transcription-polymerase chain reaction (RT-PCR)

Gene expression was measured by RT-PCR in an ExiCycler (Bioneer, Daejeon, Korea). Briefly, total RNA was isolated from 3T3-L1 adipocytes, liver and epididymal fat tissue in C57BL/6J mice using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was used for first-strand cDNA synthesis using Superscript II reverse transcriptase (BD Bioscience, Palo Alto, CA, USA). Reverse transcription was performed at 30℃ for 10 min, 42℃ for 30 min, and 99℃ for 5 min to inactivate the avian myeloblastic virus RTXL. Primers to specifically amplify the genes interested were as follows : SREBP-1c gene. forward 5′-AG CAGCCCCTAGAACAAACAC-3′. reverse 5′-CAGCAGTGA GTCTGCCTTGAT-3′ and CEBP/α gene. forward 5′-CAAGA ACAGCAACGAGTACCG-3′. reverse 5′-GTCACTGGTCA ACTCCAGCAC-3′ and PPARɣ gene. forward 5′-TCGCTGA TGCACTGCCTATG-3′. reverse 5′-GAGAGGTCCACA GAG

CTGATT-3′ and MCP-1 gene. forward 5′- GACCAGAACC AAGTGAGAT-3′. reverse 5′-TGGAAGGGAATAGTGTAATA-3′ and TNF-α gene. 5′-CGGAGTCCGGGCAGGT-3′. 5′-GCTGG GTAGAGAATGGATGAACA-3′. Amplification was performed in a master-cycler (Eppendorf, Hamburg, Germany) with denaturing at 94℃ for 1 min, annealing at 54°C for 1 min, extension at 72℃ for 30 sec for 25 cycles and finally 72℃ for 7 min. The amplified PCR products were run in 1.0% agarose gels and stained with ethidium bromide (EtBr), and visualized under UV light. The intensities of the bands were estimated by densitometry (Multi Gauge V3.0 software, Fujifilm Life Science, Tokyo, Japan) [8].

Histology

The liver and adipose tissues were fixed with 10%(v/v) buffered formalin. Tissue sections, 4 μm each in thickness, were stained with hematoxylin and eosin (H&E) priori to microscopic observation [2].

Oil red O staining

Liver tissues were fixed in 10%(v/v) buffered formalin and frozen. Tissue section were cut into 5 μm thickness, mounted on slides and allowed to dry for 1-2 hr. The slides were fixed with 10% formalin for 10 min and then the slides were rinsed with PBS (pH 7.4). After air dry, the slides were placed in 100% propylene glycol for 2 min, and stained in 0.5% Oil Red O solution in propylene glycol for 30 min. The slides were transferred to an 85% propylene glycol solution for 1 min., rinsed in distilled water for 2 changes, and processed for hematoxylin counter staining. Representative photomicrographs were captured at 200 magnifications using a system incorporated in the microscope.

Preparation of leukocytes from Liver

Livers and kidneys were perfusion, minced, placed in DMEM containing 1 mg/ml collagenase I (Sigma Aldrich) for 30 min at 37℃. The digested kidney tissue suspension was teased through a 100-μm BD Falcon cell strainer (Fisher Scientific) via a rubber end of a 1-ml syringe plunger and centrifuged at 1,200 rpm/min for 10 min. The cell pellet was washed with 2% BSA in PBS and resuspended. The pellet was then suspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ, USA), gently overlaid onto 72% Percoll and centrifuged at 2,000 rpm for 30 min at room temperature. Cells were isolated from the Percoll interface and washed twice in medium.

Flow cytometry analysis

The spleen, liver and kidney were collected. After lysis of the erythrocytes, the splenocyte, leukocytes of Liver and leukocytes of kidney were in a blocking buffer [PBS containing 2.4G2 mAb/0.2% BSA (Bovine serum albumin)/0.1% sodium azide], and then incubated with the relevant mAbs for 30 min at $4℃$. Finally, they were washed twice with staining buffer (PBS containing 0.2% BSA/0.1% sodium azide) and analyzed by FACscan (BD: Biosciences Pharmingen, Mountain View, CA).

Statistical analysis

All results obtained were analyzed in time design using the general linear model procedure of the SAS System (SAS, 1996). Data are expressed as mean ± standard deviations values. Means with different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

Results

Effects of CGJ on body weight and organ weight of diet induced obesity models

The beneficial effects of CGJ against obesity in diet induced obesity mice were investigated. To induce the obesity, high-fat diet was fed to C57BL/6 mice for 13 weeks. After obesity has been induced, mice in the CGJ group were fed CGJ containing high fat diet for another 13 weeks while mice in the ND group and HFD group were fed normal diet and high fat diet from the beginning of the experiment, respectively. Body weight gain and Food intakes of mice were shown in Table 1. Body weight of ND group was 26.4

g and that of HFD was 38.4 g after 13 weeks. Body weight gain for the ND, HFD, and CGJ group were 0.03, 0.09, and 0.01 g/day, respectively ($p<0.05$). Despite food intakes of mice were similar among 3 groups were not different, body weight gain (g/day) and final body weight of mice were significantly different ($p<0.05$). Organ weights of C57BL/6 mice fed CGJ supplemented high fat diet for 13 weeks after diet induced obesity (13 weeks with high fat diet) were shown in Table 2. The CGJ group significantly decreased liver weight than HFD group ($p<0.05$). The perirenal fat weight of ND, HFD, and CGJ group were 1.8, 2.6 and 0.2 (g/100 g body weight), respectively. But no differences were found in epididymal fat size among 3 groups.

Effects of CGJ on the plasma lipid profiles

C57BL/6 mice fed normal diet (ND) and high fat diet (HFD) for 26 weeks. High fat diet induced obesity followed a course of development which could be divided into 13 weeks and 26 weeks. This study was shown that after 13 weeks feeding, the contents of plasma TG of ND and HFD group were 88, 119 mg/dl, respectively. After 13 weeks feeding, the concentrations of plasma TC of ND and HFD group were 93, 126 mg/dl, respectively. After obesity has been induced, mice in the CGJ group were fed CGJ supplemented high fat diet for another 13 weeks. The concentrations of plasma TG and TC of C57BL/6 mice fed ND, HFD, and CGJ supplemented HFD diet were shown in Table 3. The TG concentrations of in the ND, HFD and CGJ group were 99 mg/dl, 184 mg/dl and 117 mg/dl. The TC concentrations of the ND, HFD, and CGJ group were 107, 199, and 118 mg/dl. Plasma TG and TC concentrations for the CGJ group

Table 1. Effect of Cheongukjang on changes in body weight, food intake and food efficiency ratio (FER) of DIO C57BL/6 mice

	Normal Diet	High Fat Diet +	
		HFD[1]	CGJ[2]
Body weight			
Initial weight (g)	26.4±1,1[b]	38.4±1.5[a]	38.9±2.1[a]
Final weight (g)	29.0±2.2[c]	47.1±1.7[a]	39.8±1.4[b]
Weight gain (g/day)	0.03±0.0[b]	0.09±0.0[a]	0.01±0.2[b]
Food intake (g/day) & Food efficiency ratio (FER)			
Food intake	2.65±0.4[a]	2.6±0.3[a]	2.6±0.4[a]
FER	0.01±0.0[b]	0.03±0.0[a]	0.00±0.0[c]

[1]ND: Mice fed normal diet (D12451) for 26 weeks.
 HFD: Mice fed High fat diet (D12450B) for 26 weeks.
 CGJ: High fat diet for 13 weeks to induce obesity, followed by *cheonggukjang* supplemented (10%, w/w) to the high fat diet for
    13 weeks
[2]Daily weight gain/daily weight intake
[a-c]Means with different letters in the same raw are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 2. Effect of *cheongukjang* on relative organ weight of liver, spleen, kidney, and adipose tissue in DIO C57BL/6 mice

Relative weight (g/100 g body weight)	ND	High fat diet	
		HFD[1]	CGJ[2]
Liver	4.5 ± 0.5^b	5.6 ± 0.8^a	4.1 ± 0.5^b
Kidney	1.3 ± 0.1^{NS}	0.9 ± 0.07	1.0 ± 0.14
Spleen	0.3 ± 0.04^a	0.3 ± 0.04^{ab}	0.3 ± 0.1^b
Fat pad			
Epididymal fat	3.3 ± 1.0^{NS}	4.0 ± 0.3	3.4 ± 0.2
Perirenal fat	1.8 ± 0.3^b	2.60 ± 0.2^a	0.192 ± 0.3^b

HFD: Mice fed High fat diet (D12450B) for 26 weeks.
CGJ: High fat diet for 13 weeks to induce obesity, and followed by *cheonggukjang* supplemented (10%, w/w) to the high fat diet for 13 weeks;
[a-b]Means with different letters in the same raw are significantly different ($p<0.05$) by Duncan's multiple range test.

Table 3. Effect of *cheonggukjang* on plasma lipid levels in DIO mice.

	ND		HFD		CGJ	
	13w	26w	13w	26w	13w	26w
TC (mg/dl)	92.6 ± 7.2^c	107.2 ± 27.4^c	125.8 ± 19.8^b	199.1 ± 20.9^a	125.8 ± 19.8^b	117.9 ± 7.4^b
TG (mg/dl)	88.1 ± 6^c	99.2 ± 8.1^{bc}	118.7 ± 4.8^{ba}	184 ± 19.8^a	118.7 ± 4.8^b	116.6 ± 12.4^b
ALT (Karmen/ml)	22.4 ± 2^c	31.6 ± 5.6^b	33.6 ± 1.2^b	52 ± 5.4^a	33.6 ± 1.2^b	31.6 ± 0.9^b
AST (Karmen/ml)	23.6 ± 0.5^c	36.1 ± 6.4^b	37.2 ± 1.3^b	60.6 ± 9.1^a	37.2 ± 1.3^b	36.3 ± 1.3^b
Leptin (pg/ml)	88.7 ± 5.5^d	108.5 ± 19.8^d	237.1 ± 38.3^b	308.8 ± 36.2^a	237.1 ± 38.3^b	172 ± 32.7^c
Adiponectin (μ/dl)	8.1 ± 0.2^a	7.4 ± 1.6^a	6.2 ± 0.0^b	5.4 ± 0.6^b	6.2 ± 0.0^b	7.2 ± 0.6^a
Glucose (mg/dl)	68 ± 5.4^d	96.3 ± 16.8^c	115.5 ± 12.6^b	140.9 ± 16.5^a	115.5 ± 12.6^b	73.6 ± 5.9^b
Insulin (ng/dl)	0.8 ± 0.0^d	0.9 ± 0.1^c	1.3 ± 0.1^b	2.0 ± 0.1^a	1.3 ± 0.1^b	1.3 ± 0.1^b

13w; ND: Mice fed normal diet (D12451) for 13 weeks, HFD: Mice fed High fat diet (D12450B) for 13 weeks
26w; ND: Mice fed normal diet (D12451) for 26 weeks, HFD: Mice fed High fat diet (D12450B) for 26 weeks
CGJ: High fat diet for 13 weeks to induce obesity, and followed by *cheonggukjang* supplemented (10%, w/w) to the high fat diet for 13 weeks;
[a-d]Means with different letters in the same raw are significantly different ($p<0.05$) by Duncan's multiple range test.

were significantly lower than that for the HFD group ($p<0.05$). After 13 weeks feeding, the levels of plasma AST of ND and HFD group were 24, 37 karmen/ml. The levels of plasma ALT of ND and HFD group were 22 and 34 karmen/ml. After 26 weeks feeding, the AST and ALT levels of CGJ (36, 32 Karmen/ml) were significantly lower than the HFD group (61, 52 Karmen/ml). Measured values at the end of experimental period for plasma leptin, adiponectin, glucose and insulin in C57BL/6 mice fed high fat diets containing *cheonggukjang* for 13 weeks after diet induce obesity were given in Fig. 2. The secretion of plasma leptin of ND and HFD group were 89, 237 pg/ml. After 26 weeks feeding, the leptin secretions in the ND, HFD and CGJ group were 108, 309 and 172 pg/ml, respectively ($p<0.05$). After 13 weeks feeding, the secretion of plasma adiponectin of ND and HFD group were 8.1, 6.2 μg/ml. After 26 weeks feeding, the adiponectin secretions in the ND, HFD and CGJ group were 7.4, 5.2 and 7.2 μg/ml, respectively. The glucose levels of

diet-induced obesity in C57BL/6 mice were increased to 100 mg/dl (222), which feeding high fat diet for 13 weeks. After 13 weeks feeding, the level of plasma glucose of ND and HFD group were 68, 115.5 mg/dl. In this study, mice fed CGJ supplemented high fat diet for 13 weeks after diet-induced obesity for 13 weeks. After 26 weeks feeding, the glucose level in the ND, HFD and CGJ group were 96.3, 140.9 and 73.6 mg/dl, respectively. In the HFD group, plasma glucose levels increased by approximately 46% compared with the ND group. After 13 weeks feeding, the level of plasma insulin of ND and HFD group were 0.8 and 1.3 ng/ml. After 26 weeks feeding, the insulin level in the ND, HFD, and CGJ group were 0.9, 2.0 and 1.3 mg/dl, respectively.

Effect of CGJ on liver steatosis

High fat diet has known to alter hepatic lipid accumulation and cause hepatic steatosis [26]. Given the significant improvement in plasma lipid profile by CGJ (Table 3), we

hypothesized that CGJ supplementation attenuates diet-induced obesity and hepatic steatosis. Liver of HFD group showed higher lipid droplets than those fed normal diets (ND) (Fig. 1A). Liver of HFD group were enlarged and produced a yellowish color, indicating liver steatosis. On the other hand, administration of CGJ reversed the liver to remain red and healthy. The lipid droplets were significantly lower in CGJ group than HFD group. In histology analysis, the liver of HFD mice exhibited a typical sign of fatty liver showing the accumulation of many fat droplets. To confirm the beneficial effect of *cheonggukjang* consumption on the lipid droplets was demonstrated by a histological examination of liver (Fig. 1B). The contents of lipid droplets were significantly higher in the HFD mice (135%) than CGJ mice (57%). To confirm the lipid accumulation in the liver, staining was performed using the Oil red O (Fig. 1C). In the Oil red O stained slides of the HFD group, many more red droplets within the cytoplasm of hepatocytes were present in comparison with ND and CGJ group. The induction of obesity with high fat diet was associated alterations in adipogenic transcription factor. To investigate how hepatic fat accumulation was regulated in ND, HFD and CGJ groups. The mRNA level of SREBP-1c and CEBP/α on the CGJ group were decreased by 10% and 29%, respectively, compared to that of the HFD (Fig. 1D).

CGJ modulate immune function of liver

The number of total hepatic leukocytes and accumulation of macrophage were investigated in C57BL/6 mice fed with *cheonggukjang*. (Fig. 2A). The number of CD11b$^+$F4/80$^+$ T cells on ND and CGJ groups were significantly lower than the HFD group by 6 and 73%, respectively ($p<0.05$). To determine whether immature myeloid cells are crucial in mediating inflammatory hepatic steatosis, activation and accumulation of immature myeloid cells in the liver was determined (Fig. 2B, Fig. 2C). The number of Gr-1^{int}CD11b^{high} cells on ND and CGJ groups were significantly lower than the HFD group by 65 and 75%, respectively ($p<0.05$). The Gr-1^{high}CD11b^{high} immature myeloid cells of ND and CGJ were significantly decreased by 45% and 90%, respectively, compared to that of the HFD group ($p<0.05$).

Effects of CGJ on epididymal fat pads Morphology and adipogenic transcription factor

Histological staining (H & E) revealed that the increase in body fat in HFD. The effects of *cheonggukjang* consumption on the adipocyte size was demonstrated by a histological examination of adipose tissue. The size of adipocyte in CGJ group were markedly reduced by 13.5% compared to the HFD group (Fig. 3A). We measured relative levels using cDNA synthesized with total RNA of liver by RT-PCR (Fig. 3B). The mRNA expression of PPAR-ɣ on ND and CGJ
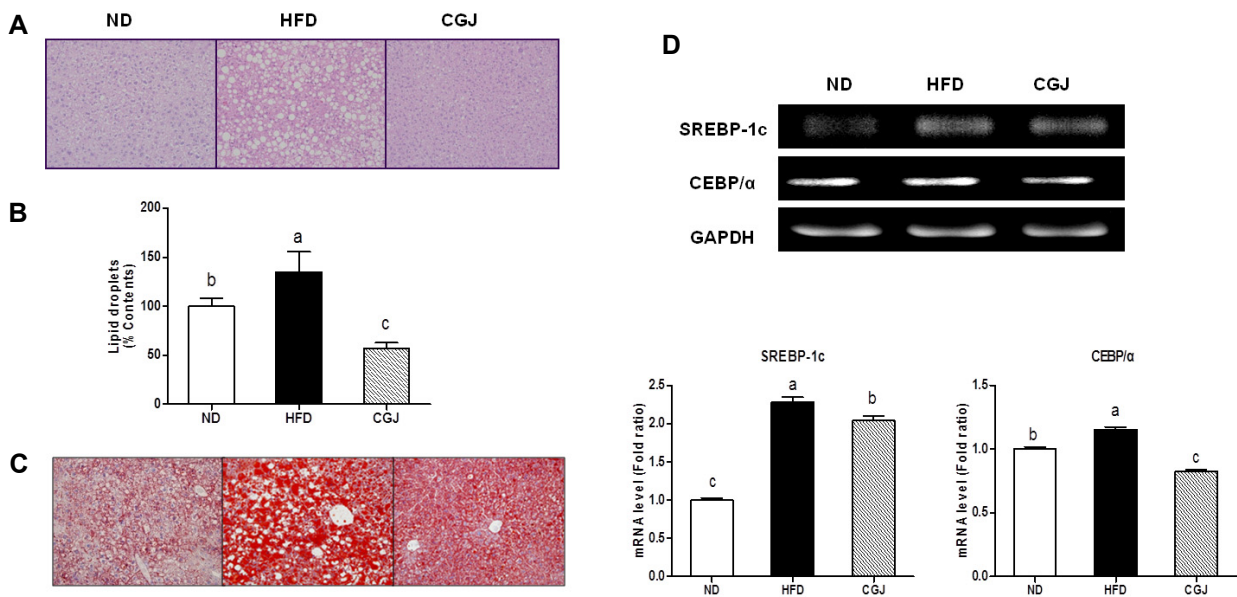

Fig. 1. Effect of resistant to steatosis of DIO C57bl/6 mice. The empty vacuole in the H&E staining and the red color droplets in Oil red O staining represent the lipid droplets in the liver. (A): Representative photomicrograph of liver sections stained with hamatoxylin and eosin(H&E). Original magnification, X200, (B): Oil Red O staining of liver. Original magnification, X200.
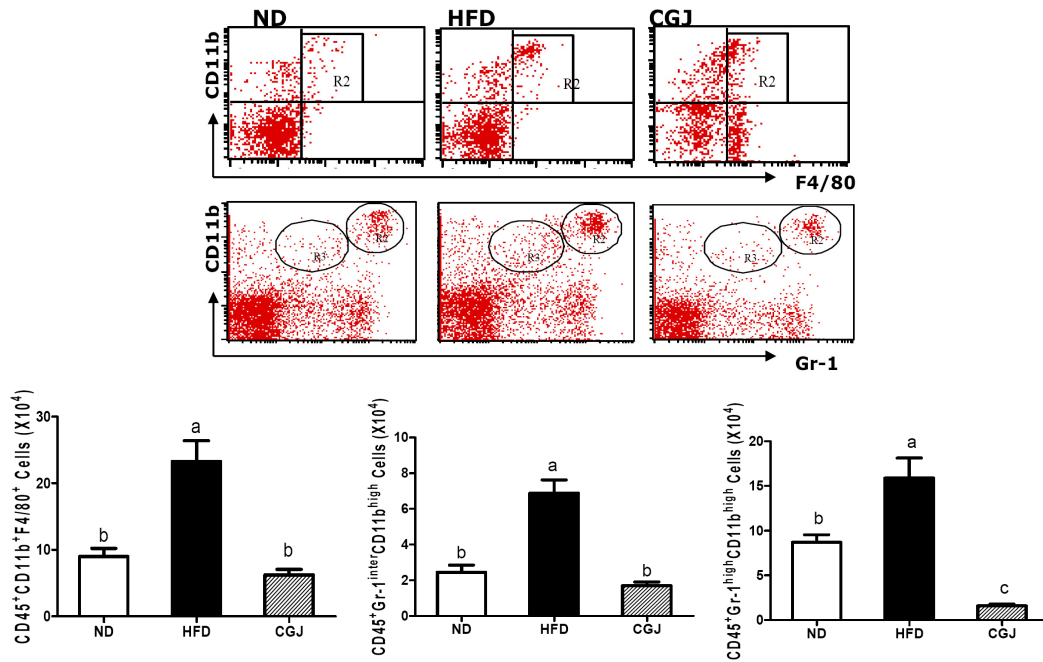
Fig. 2. Effect of *cheonggukjang* on macrophages and immature myeloid precursor cells in the liver of DIO C57Bl/6 mice. [a-c]Means with different letters in the same raw are significantly different ($p<0.05$) by Duncan's multiple range test.
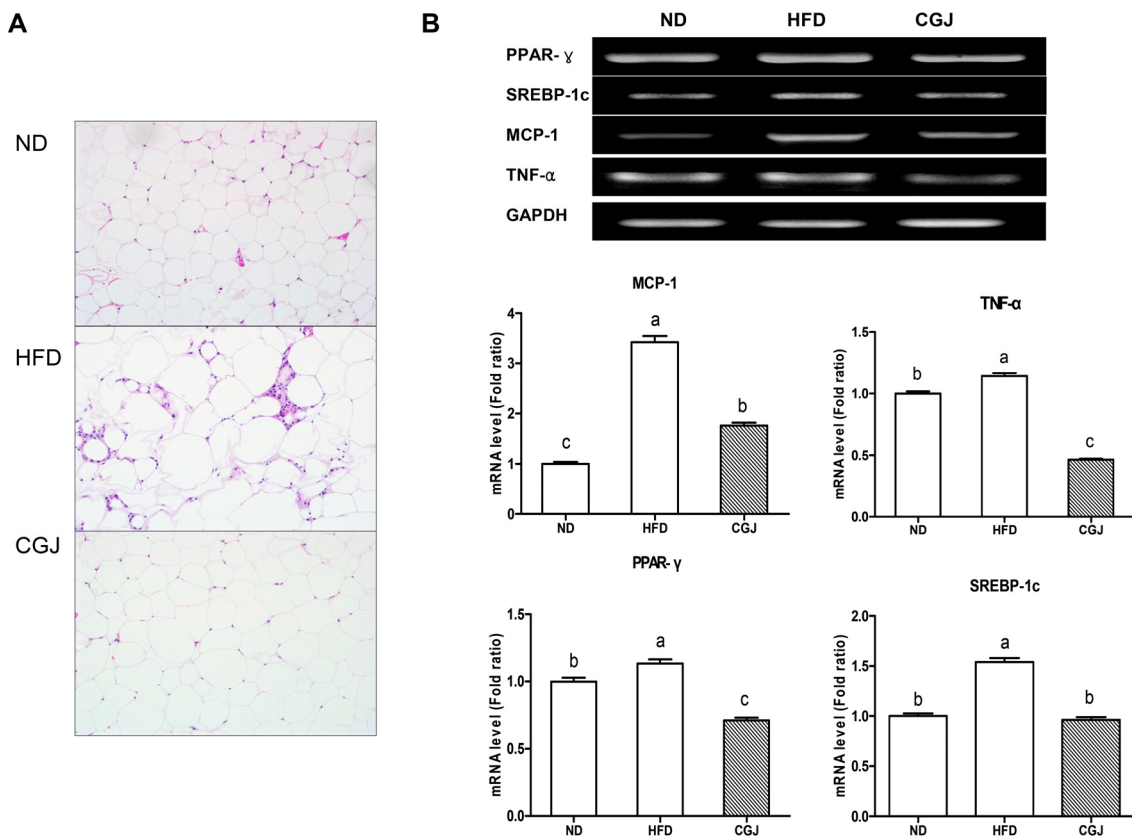

Fig. 3. Effect of *cheonggukjang* on inflammation and lipogenesis of adipose tissue in DIO C57Bl/6 mice. (A): Percent frequency distribution of adipocyte sizes indicates a shift in the size of the adipocyte population toward larger hypertrophied cells. (B): Fold ratio: Gene expression / GAPDH × ND numerical value (ND fold ration :1).

were significantly decreased by 12% and 37%, respectively, compared to that of the HFD group ($p<0.05$). The mRNA expression level of SREBP-1c on ND and CGJ were significantly downregulated by 35% and 37%, respectively, compared to that of the HFD group ($p<0.05$). Histology on epididymal fat pads from C57BL/6 mice in macrophage clusters surrounding adipocytes. To investigate whether *cheonggukjang* could decrease the expression of pro-inflammatory mediators in C57BL/6 mice. The expression of MCP-1 and TNF-α mRNA was significantly elevated in HFD group compared with those of the ND and CGJ group. Compared with the HFD group, CGJ group significantly decreased the level of MCP-1 and TNF-α mRNA by 49 and 59%, respectively ($p<0.05$). In the CGJ group, expression of TNF-α mRNA decreased by approximately 54% compared with the ND group ($p<0.05$).

Dissussion

The *cheonggukjang* is a viable candidate to improve physiological function due to its high aglycone of isoflavone. The aim of the present study was to verify the anti-obesity and anti-inflammation effects of dietary *cheonggukjang* in diet induced obese (DIO) mice. In this study, a significant reduction in body weight gain with *cheonggukjang* supplementation indicates that the *cheonggukjang* suppresses the HFD-induced increase in body weight gain and fat weight (Table 1). Despite similar food efficiency between the HFD and the CGJ groups, weight gain trends were significantly different. CGJ group indicated a dramatic decrease in the liver weight ($p<0.05$) and a marked reduction in contents of plasma TC and TG ($p<0.05$). The change in body weight and liver weight were consistent with the change in lipid profiles (Table 2, Table 3). These results suggested that *cheonggukjang* supplementation has a substantial influence on lipid metabolism in diet induced obese mice. One report acclaims that epidemically elevated levels of serum triglyceride (TG) and total cholesterol (TC) called hyperlipidemia can be achieved by administration of high-fat/cholesterol diet [47]. These data would suggest that *cheonggukjang* is a good source candidate that can reduce the risk of lipid accumulation. Because of greater fat accumulation in the liver of mice from the high fat diet, we estimated hepatic dysfunction by measuring the activities of leptin, glucose, ALT and AST into the plasma in response to membrane damage. Supplement of *cheonggukjang* downregulated levels of plasma insulin, glucose, ALT,

AST and leptin and upregulated the level of adiponectin, in spite of long term intake of high fat diet. Among the three biochemical measures, triglyceride levels exhibit significant correlation with both cholesterol and glucose [12]. The association of obesity with increased plasma insulin level [19]. High-fat diet can promote insulin resistance and alteration of insulin signaling in hepatocytes to promote increased intracellular fatty acids and eventually deteriorate hepatic steatosis [48]. Adiponectin is secreted by fat cells and circulates in the blood. The hormone suppresses glucose production in the liver and enhance glucose uptake into skeletal muscle, which is known to result from anti-atherosclerotic and insulin-sensitizing properties [43]. Promotion in expression of leptin is associated with release of pro-inflammatory cytokines [37]. Taken together, it can be proven that intake of *cheonggukjang* has beneficial effects to upregulate insulin sensitivity due to reduction of blood glucose and insulin level and to improve lipid metabolism. In addition, we may infer that the intake of *cheonggukjang* may suppress liver steatosis and proinflammatory cytokines by reduction in levels of ALT, AST and leptin and upregulation of adiponectin. Our observation of hepatic lipid droplets by H&E staining to investigate improvement of plasma lipid metabolism along with suppression of obesity-induced liver steatosis, the lipid drop in CGJ group was smaller than HFD (Fig. 1). Lipid accumulation is important mechanism in steatosis [3]. CGJ mice are protected from obesity-induced steatosis. These results would suggest that *cheonggukjang* has anti-steatosis effect as it regulates lipid profiling. The lipid droplets of liver studied was significantly correlated with their plasma TG, TC, AST, ALT, leptin, adiponectin, glucose, and insulin. These results demonstrated that administration of the *cheonggukjang* played a role in improvement of plasma lipid metabolism and liver lipid deposition. We expected that such changes may result from a certain transcriptional factor which manages regulation of lipogenesis and triglyceride synthesis, so observed expression SREBP-1c and CEBP/α. The sterol regulatory element-binding proteins (SREBPs) are transcription factors to take part in the regulation in the expression of several genes decoding enzymes for fatty acid and glucose metabolism, and they mediate the responses to changes in the nutritional status [20]. Moreover, SREBP-1c is the predominant isoform in the liver; suppression in activity of SREBP-1C by adiponectin via AMPK suppresses hepatic lipogenesis. The CCAAT/enhancer binding protein (C/EBP) is the primary adipogenic transcription

factor and their activities are initiated by synergetic trans-activation in the expressions of several adipogenic effector genes [34]. We observed the mRNA expression level of SREBP-1c and CEBP/α were increase in the HFD group ($p<0.05$), an effect significantly reversed by *cheonggukjang*. These results indicate that CGJ might have role on inhibiting the expression of transcription factors which take part in fat accumulation of liver and lipid metabolism. Body weight of ND group was 26.4 g and that of HFD was 38.4g after 13 weeks. and ND group gained 2g, HFD group 9g and CGJ group 1g after another 13 weeks. As immune cells are activated by obesity, we investigated changes in immune cells on liver steatosis upon administration of *cheonggukjang*. Macrophages plays a significant role in the pathogenesis of obesity [49]. Inflammatory reaction which involves macrophage infiltration include expression of macrophage-derived surface markers F4/80 and CD11b, both of which are also expressed on monocytes and neutrophils. Therefore, it is important to prevent accumulation of macrophage as well as accumulation of fat during obesity. Adiponectin has a crucial role in suppressing macrophage activity in the liver. These results, including reduction of CD11b+F4/80+ T cells observed in CGJ, suggested that secretion of adiponectin in CGJ were related with decreased macrophage accumulation in the liver. Also, obvious decrease in Gr-1^{int}CD11b^{high} cells and Gr-1^{high}CD11b^{high} immature myeloid cells were observed in CGJ group (Fig. 2). Neutrophils generate chemokines to recruit monocytes and dendritic cells and can determine whether macrophages can undergo differentiation to a predominantly pro- or anti-inflammatory phenotype [39]. Accumulation of immature myeloid cells may be an important component in deteriorating obesity-triggered inflammation response of the hepatic tissue, which in turn worsens metabolic morbidity including steatohepatitis [7]. These results demonstrated that long term consumption of *cheonggukjang* is associated with immune modulation effects in liver despite the consumption of a high fat diet. Diet-induced obese mice were characterized by an increase in body weight, body fat weight, and plasma leptin. In contrast, the CGJ mice decrease body weight caused principally by a highly significant decrease in perirenal fat pad and liver, although there was a trend toward a decrease in lipid parameters. We measured the size of adipocyte to investigate that such changes affect to the size of adipose tissue. Increase in adiposity has strong association with severe health problems and with an overall reduced longevity of

lifetime [27]. Size of adipose tissue for in the CGJ group was significantly lower than that for the HFD group ($p<0.05$). To investigate how lipogenesis are regulated in *cheonggukjang*, we examined expression of PPAR-γ and SREBP-1c in the adipose tissue (Fig. 3B). The peroxisome proliferators-activated receptor-γ (PPAR-γ) and sterol regulatory element-binding protein-1 (SREBP-1) are designated as key regulatory mediators of the adipogenesis process [25]. PPAR-γ is highly expressed in both brown and white adipose tissue and is observed to be a representative transcription factor in differentiation of adipocyte [35]. The mRNA level of PPAR-γ and SREBP-1c on the CGJ group was significantly decreased compared to that of the HFD group. The anti-obesity effect of CGJ might be due to inhibiting regulation promoters of adipogenic genes such as PPARγ and SREBP-1 transcription factors, resulting in inhibition of lipid accumulation by blocking adipogenesis. Inhibition of adipogenesis could be related to obesity prevention. These results suggested that CGJ reduce the (size of adipose tissue and adipogenesis transcription factor) decreases the risk of metabolic syndrome. Macrophages which invade adipose tissue play a constitutive role in deteriorating inflammatory activity accounted for characteristics of increased filtration of macrophages into white adipose tissue [49]. Thus, suppressing macrophage infiltration and accumulation induced by lipid accumulation may be helpful to reduce inflammatory reaction in case of obesity. Reduction in infiltration of macrophage by *cheonggukjang* may be helpful to suppress obesity-induced inflammation. These data indicated that there was an increase in macrophage infiltration in HFD mice but not in ND and CGJ group (Fig. 3A). As adipose tissue has elevated levels of macrophages, obesity results in local inflammation. Upon activation, macrophages secrete numbers of cytokines and chemokines, such as MCP-1 and TNF-α, that are known to cause insulin resistance in adipocytes [9, 14, 15]. Obesity is often linked to increased plasma levels of MCP-1 and overexpression in adipose tissue [15, 38]. In adipose tissue in particular, it was often seen TNF-α regulating and interfering with adipocyte metabolism at numerous sites including transcriptional regulation, glucose and fatty acid metabolism [40]. CGJ is associated with reduce inflammation of adipose tissue mediated by MCP-1 and TNF-α. This data indicated that CGJ had inhibited inflammatory action in high fat diet induced obese mice (Fig. 3B). The objective of this study was to determine the underlying mechanism by *cheonggukjang* inhibit adipogenesis. This study was

regulation of adipocytokine and suppress of liver steatosis demonstrated that *cheonggukjang* interfere with the adipogenesis process in liver and adipose tissue by down-regulating the expression of primary adipogenic transcription factors. Taken those results above, we could confirm improvement of lipid metabolism, reduction of hepatic steatosis, immune function of liver, inflammation of adipocytes and synthesis of adipose transcript factors upon intake of *cheonggukjang*. Our results determined that *cheonggukjang* decreases the lipogenic pathway and regulates immune function, and this will provide attenuation of low-grade inflammation in diet induced obese mice.

Acknowledgments

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초록 : 고지방식이로 유도된 비만 마우스에서 청국장의 항비만 및 항염증 효과

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본 연구에서는 청국장의 항비만 및 항염증 효과를 알아보고자 C57BL/6 마우스에 고지방식이를 13주감 섭취시켜 비만을 유도한 후에 고지방 식이에 청국장을 10% 첨가하여 항비만과 항염증 활성을 확인하였다. 고지방식이를 통해 비만이 유도된 mice (HFD)에 고지방식이와 함께 청국장을 첨가하여 섭취한 그룹(CGJ)의 체중 증가율은 고지방식이군과 비교하였을 때 현저하게 감소하였을 뿐만 아니라 정상식이군보다도 유의적으로 감소하였으며 혈중 TG, TC, glucose, insulin, AST와 ALT 분비는 감소하였고 adiponectin의 수치는 유의적으로 증가하였다($p<0.05$). 또한 H&E와 Oil red O staining을 통해 13주 동안 고지방식이를 통해 유도된 지방 간의 심각정도가 청국장의 섭취로 인하여 감소한 것을 확인하였다($p<0.05$). 간세포는 대식세포, T세포와 같은 면역시스템의 세포들을 포함하고 있는데 비만으로 유도된 간 조직의 염증반응에 청국장이 미치는 영향을 조사하기 위해 FACS 분석을 수행하였다. CGJ군은 HFD군에 비하여 T cell의 침윤과 대식세포의 축적, 호중구의 유입이 유의적으로 감소하였다($p<0.05$). 간 조직으로 침윤된 면역세포의 증가가 간 조직 내 염증반응을 유도하는지 확인하기 위해 염증성 cytokine의 수준을 조사한 결과 간과 지방 조직에서 염증성 cytokine인 MCP-1과 TNF-α의 mRNA 발현이 감소하였으며 지방합성에 관여하는 PPAR-γ, SREBP-1와 CEBP/α의 mRNA 발현 역시 감소하였다. 이러한 결과들을 통해 청국장이 면역시스템과 지방생성 전사 인자들의 발현에 관여함으로써 adipogenesis를 조절함과 동시에 염증 억제 효과를 가진다는 사실을 증명하였다.