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Hepatoprotective Effects of *Gardenia jasminoides Ellis* Extract in Nonalcoholic Fatty Liver Disease Induced by a High Fat Diet in C57BL/6 Mice.

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Abstract – This study was carried out to investigate the potential effects of *Gardenia jasminoides* (GJ) extracts, on hepatic steatosis and lipid metabolism in mice fed with high-fat diet (HFD). GJ extracts (100 mg/kg, × 10 weeks) fed mice showed reduced body weight, adipose tissue weight, reduced aminotransferase level in plasma and hepatic lipid (triglyceride, total cholesterol) content. These effects were accompanied by decreased expression of lipogenic genes, sterol regulatory element binding protein-1c (SREBP-1c), liver X receptor (LXR), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), cluster of differentiation 36 (CD36), lipoprotein lipase (LPL) and decreased lipogenic enzyme FAS and HMG-CoAR enzyme activities while elevating carnitine palmitoyltrasferase-1 (CPT) activity. Based on these results, we speculated that the inhibitory effect on hepatic steatosis of GJ extract containing geniposide is the result of suppression of lipid synthesis in mice fed with HFD, suggesting that GJ extract may be beneficial in preventing hepatic steatosis.
Keywords – *Gardenia jasminoides*, hepatic steatosis, high-fat diet, adipose tissue, lipogenic gene

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease which includes a spectrum of liver diseases from hepatic steatosis to nonalcoholic steatohepatitis[1-3] where the latter is known to increase the risk of liver cirrhosis and hepatocellular carcinoma.[4] NAFLD is strongly associated with metabolic syndrome, consisting of obesity, diabetes, hyperlipidemia and insulin resistance.[5] As a form of NAFLD, hepatic steatosis results from the accumulation of fat in the liver, primarily through excessive transport of free fatty acids from visceral adipose tissue into the liver and from an imbalance in *de novo* lipid synthesis and catabolism in hepatocytes.[6-8] Thus, hepatic steatosis is generally characterised by exess hepaic lipid accumulation. Several agents are known to improve NAFLD histologically or biochemically in animal and humans[9] though there are many reports on the cause of side effects.[10]

Gardenia jasminoides Ellis (GJE) have been used as a herbal medicine to treat liver and gall bladder disorders, such as hepatitis and acute jaundice, as well as inflammation and fever, in Chinese medicine for many years.[11,12,13]

A clinical study has been shown that crude *Gardenia jasminoides* extract rapidly lowers serum bilirubin and transaminase level in jaundice-induced acute hepatitis. A recent pilot study has also illustrates the effectiveness of geniposide, one component of Gardenia in reducing insulin resistance and plasma markers of liver fibrosis in patients with nonalcoholic fatty acid disease (NAFLD).[14,15] In addition, GJE has been used in traditional herbal medicine as having sedative effect. A constitution of *Gardenia jasminoides* Ellis, crocin (a glycoside of crocetin and found as crocetin in blood when taken orally)[16,17] has been reported to have anti-anxiety effect.[18] These effects in concert may have influence on the improvement of sleep quality.

In the present study, we tested the effect of the GJex containing geniposide on the development and progression of hepatic steatosis and lipid metabolism in mice fed with high-fat diet by assessing hepatic lipid accumulations, lipogenic gene mRNA expression and enzyme activities.

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Experimental

Plant materials and preparation of extracts – The fruit of *Gardenia jasminoides Ellis* (GJ) were purchased from Kyung Dong Medicinal Herb market at Seoul, Korea. The extracted powder of GJE was prepared by Lee's Biotech company and supplied for the further experiments.

Animal treatment – The male C57BL/6 mice were obtained from Koa-Tech animal company (Seoul, Korea). The animals were allowed free access to rodent chow (Purina, Korea) and tap water maintained in a controlled environment, 22 ºC and 50 ± 10% relative humidity with a 12 hr dark/light cycle, and acclimatized for at least 1 week before use. The mice were randomly divided into 3 groups; The ND (normal diet), HFD (high fat diet) and HFD + GJex (100 mg/kg) fed group. The HFD was based on diet from Open Source Diet (Central Animal Lab. Inc., Korea) containing 60 Kcal% fat while ND contains 10 kcal% fat. The diets were administered for 10 weeks, and the weight gain was measured once a week. Food intake was measured for 3 consecutive days per week by subtraction of food jar pre-and post-weights for 10 weeks. At necropsy, both side of the epididymal, retroperitoneal, perirenal adipose tissue were removed and weighed and the relative adipose tissue weight to body weight was calculated.

All animal experiments were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the institutional guidelines at Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Plasma, hepatic lipid concentrations and liver histology – After 10 weeks of GJex administration, blood samples were collected by heart puncture method to determine plasma enzyme (ALT, AST) and plasma triacylglyceride (TG), total cholesterol (TC). Plasma was prepared by centrifugation of blood (10,000 × g, 5 min, 4 ºC) and stored at −70 ºC until analysis. Plasma ALT, AST levels were measured using an automatic biochemical analyzer in the Animal Experiment Laboratory at the KRIBB. Plasma TG, TC levels were measured directly with an BCS analysis kit (Bioclinical System Coorp., Anyang, Korea). Immediately after the mice were sacrificed, the liver was removed and quickly frozen in liquid nitrogen and stored at −70 ºC.

Hepatic lipids were extracted using the modified proceduce of Folch method (1957).[19] Briefly, frozen liver tissue was homogenized in 0.9% NaCl solution and to the homogenate was added CM solution (chloroform - methanol, 1 : 2, v/v). Then the mixture were vortexed and centrifuged (2,000 × g, 20 min), and the upper phase was aspirated and filtered. The collected chloroform phase was used for analysis. Hepatic TG and TC concentrations were analyzed using an enzymatic analyzing kit (Asan Pharmaceutical Co. Ltd, Seoul, Korea).

The remaining half of the liver was removed and immediately fixed in a buffer solution of 10% formalin for pathologic analysis. Fixed tissues were processed routinely for paraffin embedding and 5 μm sections were prepared and stained with haematoxylin and eosin. Stained areas were viewed using an optical microscope.

PCR analysis for hepatic lipogenic genes expression – Total RNA from the liver was isolated and the samples were reverse-transcribed using the TOPscriptTM cDNA synthesis kit (Enzynomics, Seoul, Korea). The resulting cDNA was amplified using the PCR system (Astec, Fukuoka, Japan) and the Premix Taq (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Sequences of oligonucleotides used as primers were designed using Primer Express 3.0 (Applied BioSystems. Carlsbad, CA). All primer sets produced amplicons of the expected size, and their identity was also verified by sequencing. The cycling conditions were 95 ºC for 10 min, followed by 50 cycles of 95 ºC for 10 s and 60 ºC for 1 min. Results were normalized using the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and are represented as fold changes versus the reference gene.

Measurement of hepatic lipid-regulating enzyme activity – Hepatic enzymes were prepared according to the method of Hulcher *et al*., (1973)[20] with a slight modification. A homogenate was prepared in a buffer containing 0.1 mol/L triethanolamine, 0.02 mol/L ethylenediamine tetra-acetic acid, and 2 mmol/L dithiothreitol, pH 7.0; then centrifuged (600 × g, 10 min) to remove any cell debris; and the supernatant was again centrifuged (10,000 × g, followed by 12,000 × g, 20 min, 4 ºC) to remove the mitochondrial pellet. The supernatant was then ultracentrifuged twice (100,000 × g, 60 min, 4 ºC) to contain the cytosolic supernatant. The mitochondrial and microsomal pellets were then redissolved in 800 μl homogenization buffer and the protein content determined using the Bradford method using bovine serum albumin as the standard. FAS activity was measured in the cytosolic fraction according to the method of Nepokroeff *et al*., (1975)[7] by monitering the malonyl-CoA dependent oxidation of NADPH at 340 nm where the activity was represented by the oxidized NADPH nmol/min/mg protein. CPT activity was determined in the mitochondrial

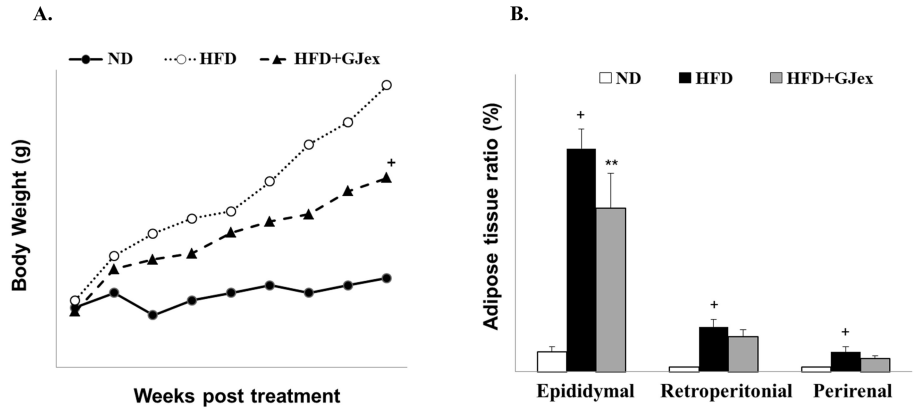

Fig. 1. Changes in body weight (A), relative fat mass (B) in C57BL/6 mice treated with normal diet (ND), high fat diet (HFD) and high fat diet + *Gardenia jasminoides* extract (HFD + GJex). Values are means ± SD (n = 6, each group). +$p < 0.01$ compared with control (ND). **$p < 0.01$ compared with the HFD treatment.

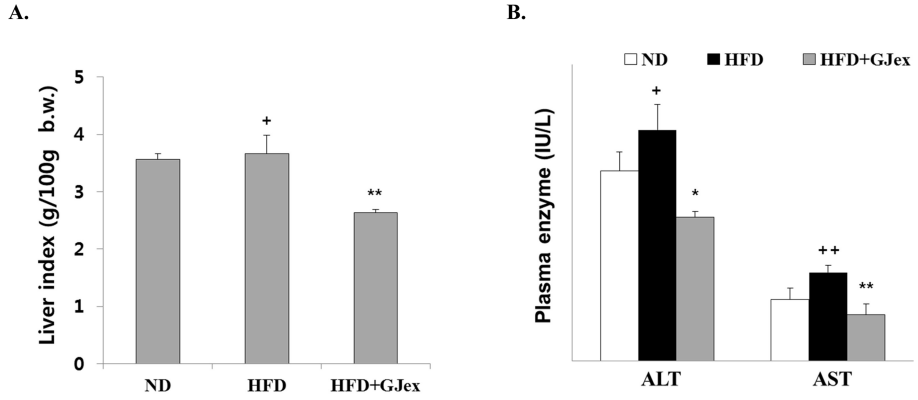

Fig. 2. Changes in liver weight (A) and plasma ALT and AST activity (B) in C57BL/6 mice treated with ND, HFD and HFD + GJex. Each value represents the mean ± SD of six mice. +$p < 0.01$, ++$p < 0.05$ compared with control (ND). *$p < 0.05$, **$p < 0.01$ compared with the HFD treatment.

fraction according to the method of Markwell[20] and the results are expressed as nmol/min/mg protein. We measured 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase activity in microsomes based on a modification of the method of Hulcher *et al*., (1973)[20]; the results are expressed as released CoA-SH nmol/min/mg protein.

Statistical analysis – All data from in vivo (n = 6) and in vitro (n = 3) experiments are given as the means ± SD. One-way analysis of variance (ANOVA) and Duncan's test were used for multiple comparisons (SPSS, version 10.0; SPSS, Chicago, IL, USA).

Results

Effects of GJex on body weight, total liver, adipose tissue weight in HFD fed mice – The body weight increased with time in HFD groups, but decreased significantly in HFD + GJex group ($p < 0.01$) (Fig. 1A). The adipose tissue ratio also decreased significantly in HFD + GJex fed mice (Fig. 1B). A significant decrease in epididymal ($p < 0.01$), retroperitoneal and perirenal weight to body weight was found in the GJex fed group. The total liver weight were decreased significantly in GJex fed HFD mice compared to the HFD mice ($p < 0.01$) (Fig. 2A).

Effects of GJex on hepatic enzyme activities, lipid contents and fatty droplet accumulations in mice – The HFD fed group (HFD) had elevated ALT and AST levels compared to ND group ($p < 0.05$, Fig. 2B) while GJex treatment attenuated HFD induced ALT, AST levels significantly ($p < 0.01$ and $p < 0.05$ each). GJex reversed the ALT, AST increase, suggesting that it has the potential to prevent liver injury.

To analyze the possible role of GJex in lipid metabolism, which is the key factor relative to fatty liver formation TG and TC were investigated. As shown in Fig. 3A and B, the increase of plasma and liver levels of TG and TC in HFD group ($p < 0.01$ and $p < 0.05$) significantly suppressed by GJex ($p < 0.05$ at plasma TG; $p < 0.01$ at liver TG and

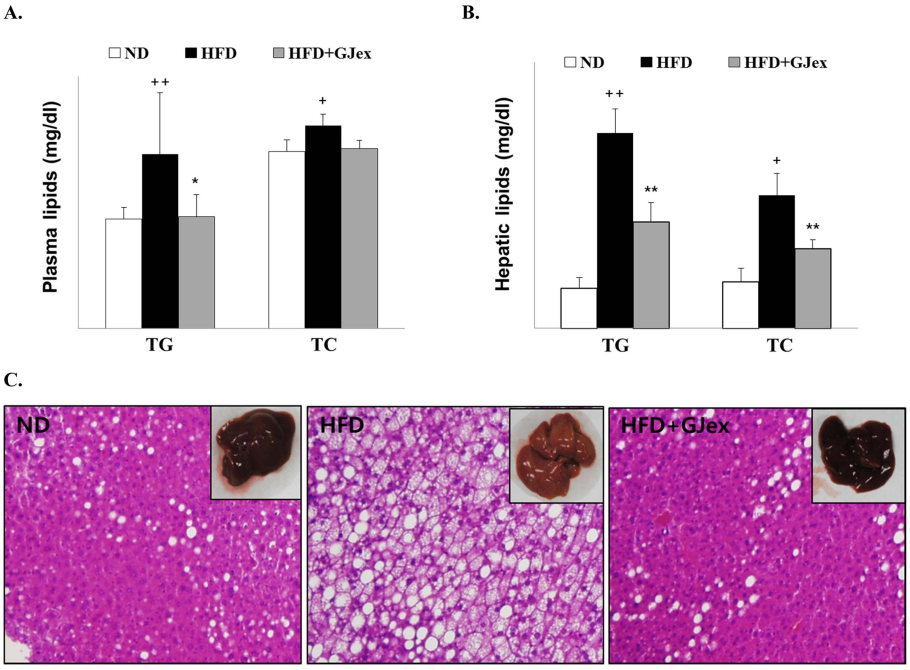

Fig. 3. Plasma (A), hepatic lipid (B) and representative photomicrograph of fat droplet (C) in C57BL/6 mice with ND, HFD and HFD + GJex. Values are mean ± SD (n = 6, each group). +$p < 0.01$, ++$p < 0.05$ compared with control (ND). *$p < 0.05$, **$p < 0.01$ compared with the HFD treatment. TG: triglyceride, TC: total cholesterol. Arrows indicate the microvesicular-type fat in the cytoplasm of the hepatocytes. Representative photomicrographs showing the inhibition of fatty liver in GJex-administead mice. H&E staining, × 200.

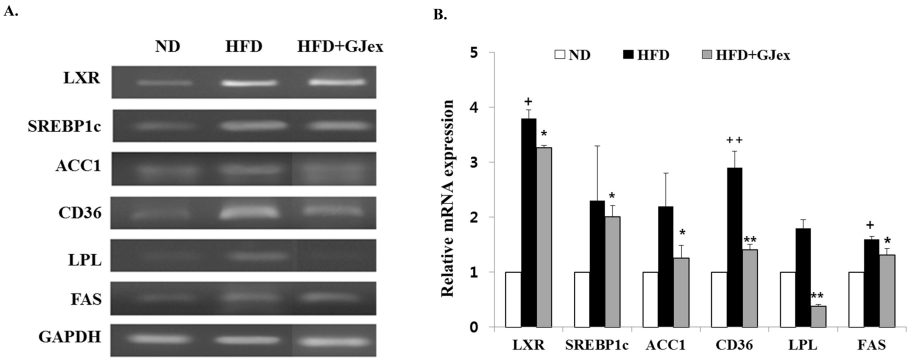

Fig. 4. Hepatic expression of lipogenic genes in C57BL/6 mice. RT-PCR was used for mRNA expression of lipogenic genes using primers as indicated on Table 1(A). The relative levels of specific mRNA levels were presented (B). Values are means ± SD (n = 6 each group). +$p < 0.01$, ++$p < 0.05$ compared with control (ND). *$p < 0.05$, **$p < 0.01$ compared with the HFD treatment. LXR: liver receptor X, SREBP-1c: sterol regulatory element binding protein-lc, ACC: acetyl Co-A carboxylase, CD36: cluster of differentiation 36, LPL:lipoprotein lipase, FAS: fatty acyl synthase, GAPDH: glyceraldehyde-3 phosphate dehydrogenase.

TC). Representative photomicrographs of liver histology was shown in Fig. 3C. HFD fed control mice showed a high accumulation of microvesicular-type fat in the cytoplasm of the hepatocytes and showed focal hepatitis charadterized by scattered inflammatory cells and of inflammatory foci. Treatment of GJex clearly improved the miceovesicular hepatic steatosis (Fig. 3C, HFD + GJex), almost the same levels as ND liver histology (Fig. 3C, ND). These data clearly suggest that the GJex can prevent hepatosteatosis via down regulating the accumulation of lipid in liver cells.

Effect of GJex on hepatic lipid regulatory gene expressions in mice – To investigate the underlying mechanism responsible for the inhibitory effect of GJex on HFD-induced hepatic steatosis, we determined hepatic expression levels of lipogenesis-regulating genes, including LXR, SREBP-1c, ACC1, CD36, LPL and FAS. Glyceral-delycle-3-phosphate dehydrogenase (GAPDH) was used as a control (Fig. 4). Compared with the HFD group, mice treated with GJex showed significantly reduced mRNA

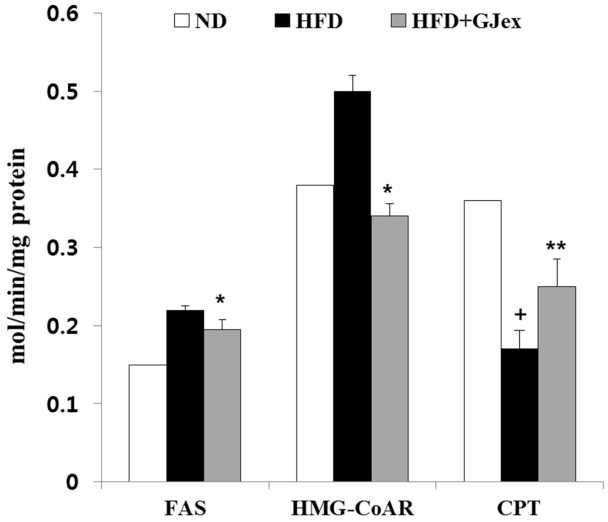

Fig. 5. Acitvity of hepatic lipid regulating enzymes; fatty acid synthase (FAS), carnitine palmito-transferase (CPT) and 3-hydroxy-3 methylglutamyl-coenzyme reductase (HMG-CoAR) in C57BL/6 mice treated with ND, HFD and HFD + GJex. Values are means ± SD (n = 6, each group). +$p < 0.01$ compared with control (ND). *$p < 0.05$, **$p < 0.01$ compared with the HFD treatment.

expression of the tested genes (*$p < 0.05$, **$p < 0.01$ compared to the HFD treated mice) (Fig. 4A). The relative levels of specific mRNA levels were shown (Fig. 4B).

Effects of GJex on hepatic lipid-regulating enzyme activities in mice – As representative enzymes, mediating lipid biogenesis and lipid degradation, the activity of FAS, HMG-CoA reductase and CPT was assessed to investigate whether GJex would affect the enzyme activities of lipid metabolism in the liver (Fig. 5). Hepatic FAS and HMG-CoA reductase activity was significantly decreased ($p < 0.05$ each), whereas CPT activity was increased in the GJex-fed group compared to the HFD-fed mice group ($p < 0.01$).

Conclusions

Hepatic steatosis is emering as the most important cause of chronic liver disease associated with the increasing incidence of obesity.[22] It can progress to nonalcoholic steatohepatitis in 10% - 20% of patients[23] and even to advanced cirrhosis and hepatocellular carcinoma.[24] In this study, we showed that the administration of GJex protected against the development and progression of hepatic steatosis induced by a HFD in C57BL/6 mice. Moreover, the reductions in hepatic lipids (TG, TC) and plasma lipids (TG, TC) were observed in GJex-administered C57BL/6 mice.

The inhibitory effect of GJex on hepatic steatosis appeared to be related to the suppression of lipogenesis enzyme activity and the acceleration of fatty acid oxidation in HFD-fed mice. But the other possibilities of GJex effect on hepatic steatosis, like intestinal lipid absorption, pancreatic lipase activity, modulation of fecal sterol excretion by GJex treatment should be further considered. Significant reduction of body weight showed in HFD + GJex groups compared to the HFD treated group (Fig. 1A) though the amount of food consumption was similar between the groups (data not shown). These results suggested that suppressed lipid production and increased energy expenditure were involved in preventing hepatic steatosis in GJex-fed mice.

To further investigate the reduced hepatic lipid production in GJex-fed mice, we explored the mRNA expression of lipogenic and lipid-regulating enzymes, including SREBP-1c, FAS, LXR, ACC1, LPL and CD36 using quantitative PCR. LXR is a member of a nuclear receptor superfamily that regulates the expression of key proteins involved in lipid metabolism and inflammation, serving as a kind of nuclear cholesterol sensor.[25]

LXR also increase the expression of SREBP-1c, which leads to increased hepatic triglyceride synthesis. Our experimental results showed that LXR expression is decreased in GJex-fed mice. SREBP-1c is a key transcription factor regulating the expression of enzymes involved in lipogenesis and fatty acid desaturations as well as in response to fat and insulin.[26] Its expression was significantly higher in NAFLD, nearly 5-fold greater than the controls. SREBP-1c is positively regulated by insulin signaling pathways, including insulin receptor substrate 1 and 2. It is known that SREBP-1c is negatively regulated by AMP-activated protein kinase (AMPK).[27] Their experimental results showed that in NAFLD, insulin signaling via IRS-1 causes the up-regulation of SREBP1-c, leading to the increased synthesis of fatty acids by the hepatocytes.[28]

In this study, significant suppression of SREBP-1c and LXR mRNA by GJex were confirmed, which in turn would be expected the down regulation of lipogenic genes such as FAS and ACC. In our experimental data also firmly confirmed the down-regulation of FAS and ACC in GJex-fed group which are involved in fatty acid biosynthesis. LPL, the HMG-CoA reducase catalyze the reduction of HMG-CoA to CoA and mevalonate, the rate-limiting reaction in the de novo synthesis of cholesterol. Hepatic mRNA expression levels of HMG-CoA reductase is important in cholesterol metabolism. Our experimental results showed the significant decrease of the HMG-CoA reductase expression in GJex –fed mice. In contrast

hepatic activity of CPT an enzyme involved in fatty acid β oxidation was increased while FAS and HMG-CoAR were decreased in GJex-fed mice.

In conclution, the present study showed that GJex inhibits hepatic steatosis and has plasma lipid-lowering effect in HFD-fed C57BL/6 mice. These effects are mediated by the down regulation of hepatic genes involved in lipogenesis and modulation of lipid metabolism-related enzyme activity.

These results suggested that *Gardenia jasminoides Ellis* is useful as a potential dietary food supplier for intervention of hepatic steatosis and hyperlipidemia.

Acknowlegements

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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