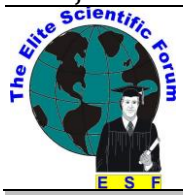
Pak. j. life soc. Sci. (2019), 17(2): 58-67 E-ISSN: 2221-7630; P-ISSN: 1727-4915



Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Anti-Obesity Properties of Jin-Chang-Mi in High-Fat Diet-Fed C57BL/6 Mice

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ARTICLE INFO
Received: Dec 2, 2019
Accepted: Dec15, 2019

Keywords
Anti-obesity
Fatty liver
Garcinia gummi-gutta
High-fat diet
Jin-chang-mi
White adipose tissue

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ABSTRACT

Obesity is a potential source of various chronic diseases such as type-2 diabetes, hypertension and hyperlipidemia. This study aimed to investigate the effects of Jin-chang-mi (JCM) and its synergistic effect with *Garcinia gummi-gutta* (GG) on high-fat-diet (HFD)-fed obese mice. Four-week-old male C57BL/6J mice (n=36) were divided into 6 groups viz. Normal, HFD, JCM320, JCM320 + GG150, JCM640 + GG150 and GG150). The mice of all the groups were fed HFD for 3 weeks to induce obesity, followed by oral administration of different treatments daily for 7 weeks along with HFD. The mice were euthanized 24 h after the final administration of different treatments. Blood, liver, and white adipose tissue samples were collected from each mouse for further analysis including serum chemistry, hematoxylin and eosin staining, and real-time PCR. The administration of JCM, GG, or their combination partially inhibited body weight gain and feed efficiency; and reduced the levels of serum glucose and glutamic pyruvic transaminase. The accumulation of white adipose tissue was significantly inhibited in the GG150 group. There were no significant differences in the expression levels of genes related to the browning of white adipose tissue among different treatment groups. Liver size and weight were markedly increased by the HFD group while mice restored the liver size and weight following sample treatment (especially, the JCM320 and JCM320 + GG150 groups). In conclusion, oral administration of JCM with GG exhibited slight anti-obesity effects by reducing body weight gain (accumulation of adipose tissue) and suppressing the progression of hepatic steatosis by attenuating micro- and macro-vesicular lipid accumulation.

INTRODUCTION

Obesity is a significant public health problem worldwide owing to increased food availability, lifestyle changes, nutritional imbalance and/or viral infection (Saltiel, 2016). Obesity, defined as excessive fat accumulation, results from an imbalance in calorie absorption and expenditure, which can raise the risk of disorders including type-2 diabetes, hyperlipidemia, hyperglycemia, hypertension, coronary heart disease, atherosclerosis, stroke and even a few types of cancer and infertility (Lee et al., 2018; Hu, 2008).

The treatment for obesity includes a two-step process, which consists of assessment and management. For assessment, several factors are needed to evaluate the disease status. Among them, increased serum low density lipoprotein (LDL) cholesterol and decreased serum high density lipoprotein (HDL) cholesterol levels might be related to the presence of cardiovascular diseases in obese people (Lyznicki et al., 2001). Additionally, non-alcoholic fatty liver disease (NAFLD) is also commonly related to obesity. Glutamic oxaloacetic transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT), indicators of liver damage, are commonly evaluated to determine the liver function. Moreover, the levels of creatinine and BUN are important biomarkers to determine renal failure, which is one of the complications of obesity (Wang et al., 2008; Baum et al., 1975).

Adipocytes in white adipose tissue (WAT) lack mitochondria and energy is stored as triglycerides. On the other hand, adipocytes in brown adipose tissue (BAT) have a high amount of mitochondria and thus are able to consume stored energy to regulate body temperature (Luna-Luna et al., 2015). The BAT is characterized by high metabolic activity due to the presence of uncoupling protein (UCP-1) which is located on the inner mitochondrial membrane (Tsubone et al., 2005; Spiegelman and Flier, 1996). The UCP-1 is also regulated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (Ringholm et al., 2013). Some previous studies also revealed browning of WAT into beige adipose tissue (Bartelt and Heeren, 2014; Nedergaard and Cannon, 2014). The increased expression levels of UCP-1 and PGC-1α in WAT suggested an increase in energy consumption and the browning of WAT, thus indicating a reduction of WAT in the body (Bartelt and Heeren, 2014).

Currently, different pharmacologically active compounds like orlistat, phentermine, topiramate and lorcaserin are potential therapeutic agents to manage the obesity (Saltiel, 2016). Although these pharmacological drugs have shown positive effects but each with some associated side effects including GI disturbance, hypertension, tachycardia, and headache. Therefore, there have been extensive studies on the use of natural products as a substitute method for the prevention and management of obesity.

In this regard, *Garcinia gummi-gutta* (GG), commonly known as *Garcinia cambogia*, is a widely marketed natural dietary supplement and potential anti-obesity agent. It has inhibitory effects on fat synthesis, body weight gain and appetite (Semwal et al., 2015). Jin-chang-mi (JCM) is rice (*Oryza sativa L.*) that has been stored for 3–5 years, which has been used in Korean oriental medicine to treat fever, diarrhea, vomiting, edema, and abdominal disturbance without toxicity effects. When rice is converted into old rice, it is less edible due to discoloration and a strong unpleasant odor, accompanied by incidental problems such as maintenance cost for storage. According to the Korean government, rice stock has reached 1.9 million tons in 2015, 2.36 million tons in 2016, and 2.44 million tons in 2017 (kipo.go.kr). Therefore, the development of dietary supplements using JCM can increase the value of old rice and reduce economic losses by promoting the consumption of old rice.

In this study, we investigated the potency of JCM and its synergistic effects with GG on high-fat diet (HFD)-fed obese mice by examining body weight changes, serum markers, WAT accumulation, browning gene expression in WAT; and histological changes in the adipose tissue and liver.

MATERIALS AND METHODS

Preparation of JCM

JCM was provided by Daedong Korea Ginseng Co (Geumsan, Republic of Korea). White rice, which is more than 3 years old, was washed and dried. The dried rice was subjected to thermal treatment at 180 °C for 20 min and ground to 100 mesh or less to prepare rice powder. The resulting powder was then weighed accordingly for oral administration.

Animal experimentation

Four-week-old male mice (C57BL/6J; n=36) were obtained from Orient Bio Inc. (Seongnam, Republic of Korea) and acclimatized for one week in environmental controlled air-conditioned room and 12-h light/dark cycle, with temperature and humidity of 22±2°C and 50 ± 10%, respectively. Feed and water were provided *ad libitum* throughout the study. The mice were fed HFD (D12492; Research Diets, NJ, USA) for 10 weeks. Normal mice were provided a standard normal diet (AIN-76A; Hyochang Science, Republic of Korea). The diets composition is given in Table 1. After 3 weeks of HFD consumption, the average body weight of HFD-fed mice was about 20% greater than normal diet-fed mice. From the 4th week onward, the mice were divided into one of the following 6 groups (n=6): (1) Normal control group fed a normal diet alone; (2) HFD group fed a HFD alone; (3) Rest of the groups were fed HFD along with various treatment combinations as described in Figure 1. Each sample was orally administered once a day for 7 weeks. Mice in the normal control and HFD groups were orally administered with distilled water, which was used as a sample vehicle. Feed intake and body weights were measured on weekly basis. Feed efficiency (%) was calculated as follows: [body weight gain (g)/feed intake (g)] × 100.

Table 1: Composition of experimental diets

Diet	AIV-76A		D12492	
	gm%	Kcal%	gm%	Kcal%
Protein	20.3	20.8	26	20
Carbohydrate	66	67.7	26	20
Fat	5	11.5	35	60
Kcal/gm	3.9		5.24	
Ingredient	gm	Kcal	gm	Kcal
Casein	200	800	200	800
L-Cystine	0	0	3	12
Corn Starch	150	600	0	0
DL-Methionine	3	12	0	0
Maltodextrin 10	0	0	125	500
Sucrose				
Cellulose	50	0	50	0
Corn Oil	50	450	0	0
Soybean Oil	0	0	25	225
Mineral Mix	35	0	10	0
Vitamin Mix	2	0	10	40
Choline Bitartate	2	0	2	0

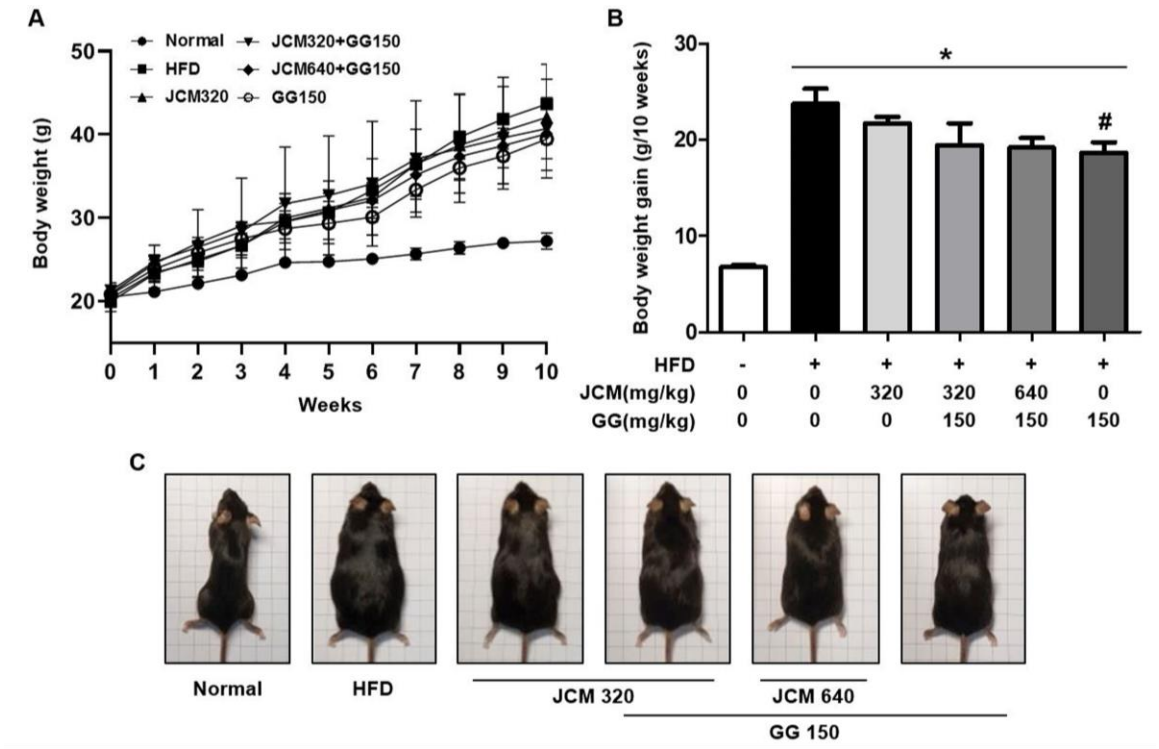

Fig. 1: Induction of obesity and the effects of JCM, GG, and their combination on the body weight of HFD-fed mice. (A) The body weight was recorded weekly for 10 weeks. The (B) total body weight gain and (C) body size of mice were examined. HFD: high-fat diet; JCM: Jin-chang-mi; GG: Garcinia gummi-gutta. Values are presented as the mean±SD (n=6). *P<0.05 versus the normal group; #P<0.05 versus the HFD group.

Serum chemistry

Mice were euthanized following an overnight fasting 24 h after the final administration of JCM and GG. Blood was collected through cardiac puncture and transferred into BD Vacutainer® (BD, Plymouth, UK). The tubes were then centrifuged at 3,000 rpm for 15 min. The serum was collected from the supernatant, and the total cholesterol (T-CHOL), glucose, LDL, HDL, GOT, GPT, CRE, and BUN levels were determined using a chemistry analyzer (Model and make).

Tissue collection and Histological Examination

Organs (liver, kidney and spleen) and WAT (subcutaneous, epididymal, mesenteric and perirenal fat) were collected from the mice and weighed immediately. Liver and epididymal adipose tissues were fixed in 10% neutral formalin for hematoxylin and eosin (H&E) staining. After dehydration, the tissues were embedded in paraffin, cut into sections, and stained with H&E. To quantify the adipocytes, the number of adipocytes was counted from randomized 100× micrographs of epididymal adipose tissue using the software AdipoCount (Zhi et al., 2018).

Real-time polymerase chain reaction (PCR)

Epididymal WAT and BAT were harvested from the mice. Total RNA was extracted from the harvested adipose tissue using TRIzol solution. Isolated RNA was reversely transcribed into cDNA. The UCP-1 and PGC-1α genes were amplified using SYBR® Green (Thermo Fisher Scientific, UK) in the C1000™ Thermal Cycler (Bio-Rad, USA). The sequences of the primers used in the study are shown in Table 2. PCR conditions were as follows: 1 cycle of 95℃ for 5 min, 41 cycles of 95℃ for 15 sec, 60℃ for 15 sec and 72℃ for 15 sec followed by 1 cycle of 95℃ for 10 sec, 65℃ for 5 sec and 95℃ for 5 sec. The relative expression levels of the genes were normalized against the expression of the housekeeping gene GAPDH.

Statistical analysis

Statistical significance was analyzed by one-way ANOVA with Dunnett's post-test using GraphPad Prism version 5 (San Diego, CA, USA) and data were presented as the mean±standard deviation (SD). P<0.05 was considered as significant.

RESULTS

Inhibitory effects of JCM and GG on the body weight gain in HFD-fed obese mice

Obesity was successfully induced in mice following 3 weeks of HFD feeding. Mice were orally administered with or without samples for 7 weeks. As shown in Figure 1, the body weight and size of the HFD fed

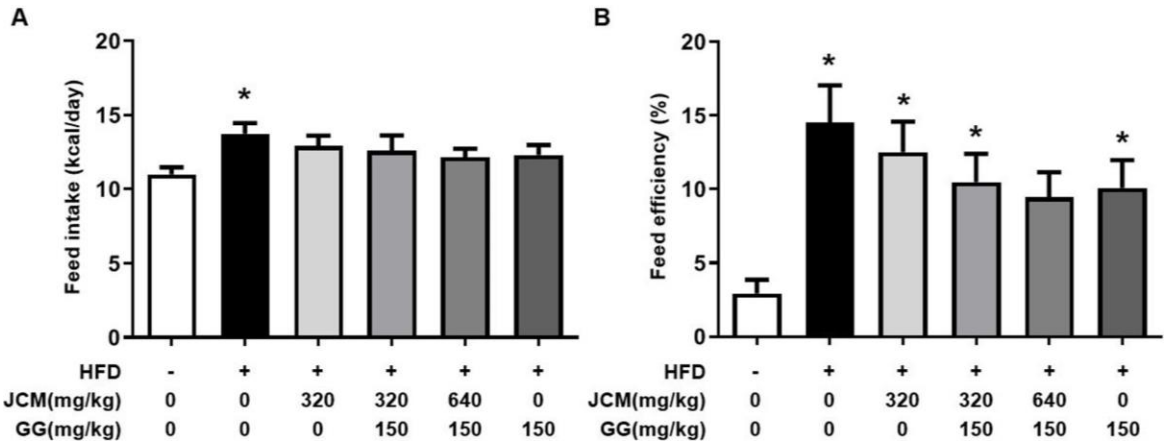

Fig. 2: Effects of JCM, GG, and their combination on (A) feed intake and (B) feed efficiency rates in HFD-fed mice. Feed intake was measured weekly for 10 weeks. The FER was calculated as (body weight gain (g/day) / feed intake (g/day)) × 100. HFD: high-fat diet; JCM: Jin-chang-mi; GG: *Garcinia gummi-gutta*. Values are presented as the mean ± SD (n = 6). **P* < 0.05 versus the normal group; *#P* < 0.05 versus the HFD group.

Table 2. Primer sequences used in real-time PCR analysis

Gene	Primer	Sequence (5′→3′)
PGC-1α	Forward	AAGACAGGTGCCTTCAGTTCACTCTCAG
	Reverse	AGCAGCACACTCTATGTCACTCCATACAG
UCP-1	Forward	ACTGCCACACCTCCAGTCATT
	Reverse	CTTTGCCTCACTCAGGATTGG
GAPDH	Forward	CACTCACGCAAATTCAACGGCAC
	Reverse	GACTCCACGACATACTCAGCAC

group were substantially increased as compared to those of the normal group. The GG150 group demonstrated the strongest reduction in body weight gain, followed by the JCM640 + GG150 group. The increase in body weight induced by the HFD was slightly decreased in all sample groups, with only the GG150 group showing a significant decrease (P>0.05).

Effects of JCM, GG and their combination on feed efficiency in HFD-fed mice

The feed efficiency ratio (FER) indicates the efficiency of feed converted into body weight. Therefore, the reduction in the FER following sample administration indicates the potential of reducing weight gain. Based on our findings, the average calorie intake per day between the groups had no significant differences (P>0.05), suggesting that treatment with our samples did not alter the appetite of the mice (Figure 2A). However, feed efficiency was strongly increased in the HFD group, and it was lower in all treated groups (Figure 2B). The JCM640 + GG150 group showed the strongest inhibition of the FER, followed by the GG150 group (P>0.05). Therefore, the treatment of the JCM640 + GG150 had an inhibitory effect on body weight gain from feed intake, which was similar in the GG150 group.

Effects of JCM, GG and their combination on the serum markers in HFD-fed mice

The HFD increased the levels of T-CHOL, glucose, HDL, GOT, and GPT and reduced CRE and BUN in the serum of mice (Fig 3). Serum glucose level was decreased in the JCM320 + GG150, JCM640 + GG150 and GG150 groups. The level of LDL was decreased in the GG150 group when compared with the HFD group (P>0.05). The mice in the JCM320+GG150 and JCM640+GG150 groups showed a slightly reduced serum GOT level, which was induced by the HFD. Moreover, the increased GPT level induced by the HFD was down-regulated in all the sample-treated groups; however, the results were not statistically significant. As GOT and GPT are liver damage markers, the results indicated that treatment with both JCM and GG attenuated liver damage induced by the HFD. However, no significant difference was observed in serum T-CHOL, HDL, CRE, and BUN in the sample-treated groups compared with the HFD group. As CRE and BUN are markers of kidney failure, the results indicated that there was no noticeable kidney damage following sample treatment.

Effects of JCM, GG, and their combination on the adipogenesis and adipocyte hypertrophy in HFD-fed mice

High fat diet markedly induced the accumulation of subcutaneous, epididymal, perirenal, and mesenteric WAT (Figure 4A). The treatment received by the GG150 group significantly inhibited the accumulation of subcutaneous and epididymal WAT (P>0.05). In addition, the amount of subcutaneous WAT was decreased in both the JCM320 + GG150 and JCM640 + GG150 groups. However, no significant difference was observed on perirenal and mesenteric WAT among the treated groups compared with the HFD group. In terms

of the total WAT weight, only the GG150 group showed a significant decrease in WAT, which was induced by the HFD (Figure 4B). The histological analysis of epididymal WAT stained with H&E demonstrated that a HFD triggered the progression of adipocyte hypertrophy and macrophage infiltration (Figure 4C). To quantify the hypertrophy of adipocytes, the number of adipocytes was counted using the software AdipoCount (Figure 4D). The adipocyte number was significantly reduced in the HFD group, which indicated that a HFD had induced the progression of adipocyte hypertrophy ($P>0.05$). Treatment with all samples slightly attenuated adipocyte hypertrophy, as demonstrated by the higher number of adipocytes compared with the number of the HFD group.

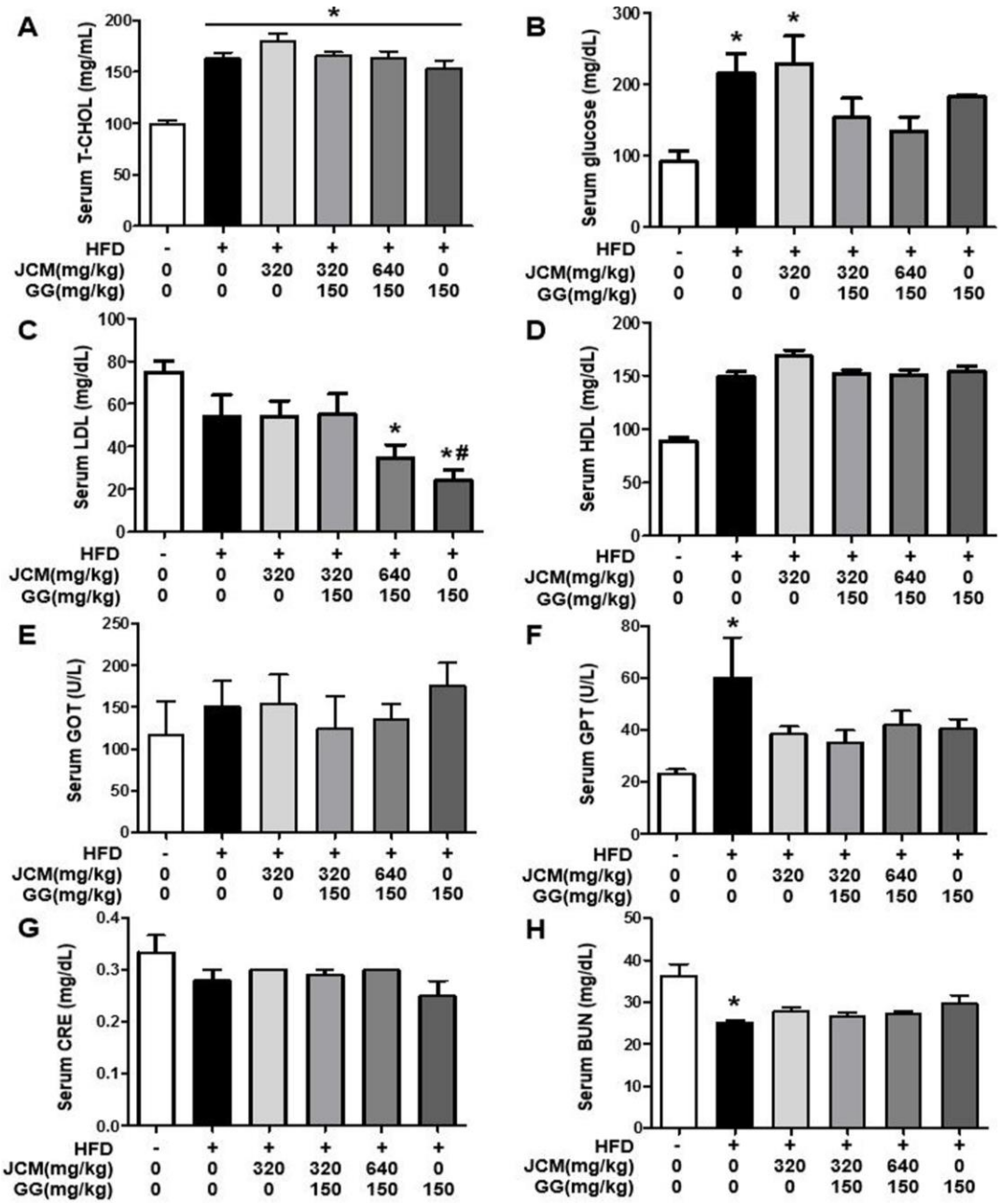

Fig. 3: Effects of JCM, GG, and their combination on the serum markers in HFD-fed mice. Blood was collected by cardiac puncture, and serum was isolated from the blood to analyze (A) T-CHOL, (B) glucose, (C) LDL cholesterol, (D) HDL cholesterol, (E) GOT, (F) GPT, (G) CRE, and (H) BUN using a blood analyzer. Values are presented as the mean ± SD (n=6). **P*<0.05 versus the normal group; #*P*<0.05 versus the HFD group.

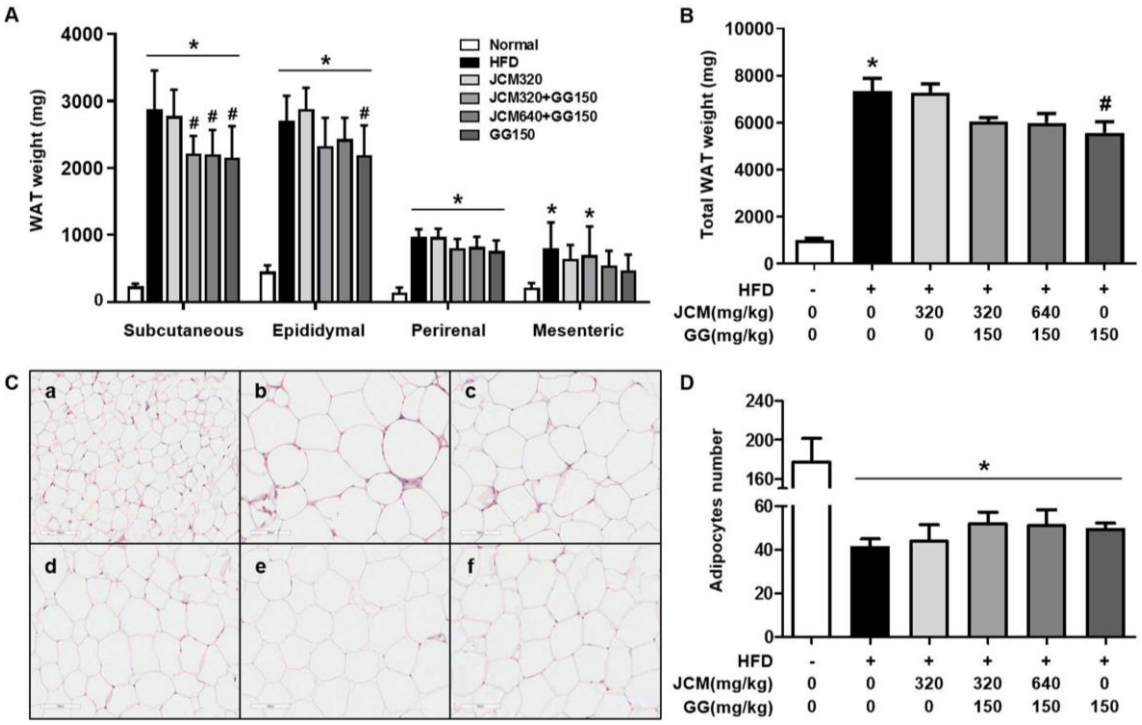

Fig. 4: Effects of JCM, GG, and their combination on the accumulation of WAT and the hypertrophy of adipocytes. (A) Subcutaneous, epididymal, perirenal, and mesenteric adipose tissues were collected from mice and weighed immediately. (B) The total adipose tissue weight per mouse was also determined. (C) Epididymal adipose tissue was stained with H&E and analyzed under a light microscope at 100× magnification. (a: Normal; b: HFD; c: JCM320; d: JCM320 + GG150; e: JCM640 + GG150; f: GG150). The scale bar presents 100μm. (D) Adipocytes were quantified by counting the number of cells in random 100 × images using the software AdipoCount. HFD: high-fat diet; JCM: Jin-chang-mi; GG: *Garcinia gummi-gutta*; WAT: white adipose tissue. Values are presented as the mean ± SD (n = 6). **P*<0.05 versus the normal group; #*P*<0.05 versus the HFD group.

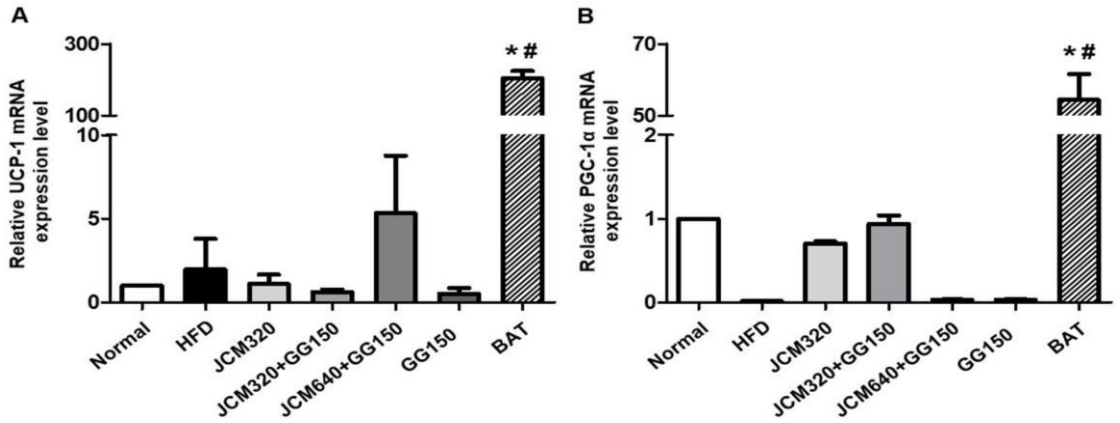

Fig. 5: Effects of JCM, GG, and their combination on the expression levels of browning genes in epididymal adipose tissue. (A) UCP-1 and (B) PGC-1α expression levels were investigated by real-time PCR. The relative expression levels of UCP-1 and PGC-1α were normalized against GAPDH expression. BAT was harvested from normal mice as a positive control. HFD: high-fat diet; JCM: Jin-chang-mi; GG: *Garcinia gummi-gutta*. BAT: brown adipose tissue. Values are presented as the mean ± SD (n = 6). **P*<0.05 versus the normal group; #*P*<0.05 versus the HFD group.

Effects of JCM, GG, and their combination on the browning of adipocytes in epididymal WAT

As shown in Figure 5A&B, all treated groups did not show a significant elevation in both UCP-1 and PGC-1α expression levels in epididymal WAT compared with BAT in normal mice (P>0.05). Therefore, our results indicated that both JCM and GG did not induce the browning of WAT.

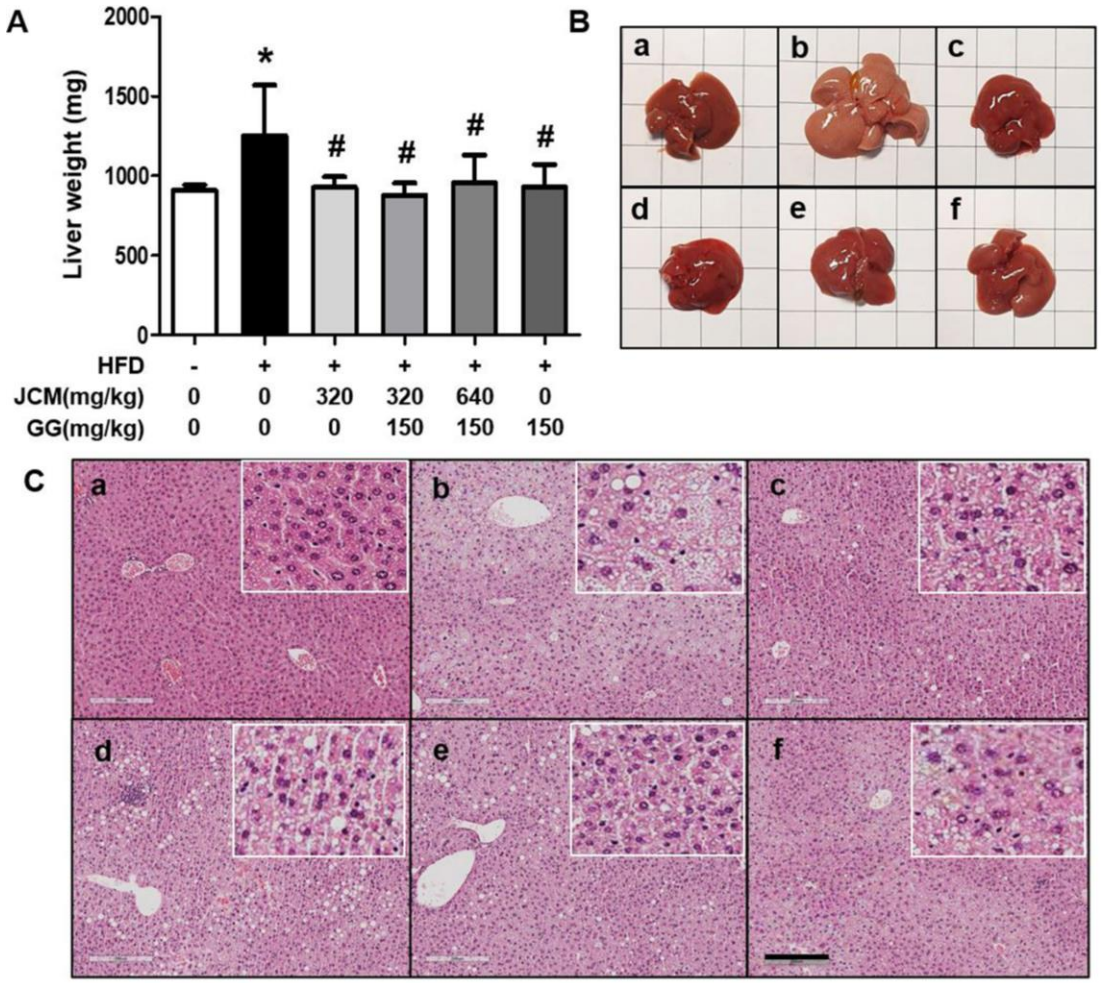

Fig. 6: Effects of JCM, GG, and their combination on the accumulation of lipids in the liver of HFD-fed mice. The (A) mean liver weight, (B) representative images of the gross liver, and (C) histological images of the H&E-stained liver in each group are shown (a: Normal; b: HFD; c: JCM320; d: JCM320 + GG150; e: JCM640 + GG150; f: GG150). The scale bar presents 200 µm. HFD: high-fat diet; JCM: Jin-chang-mi; GG: *Garcinia gummi-gutta*. Values are presented as the mean ± SD (n = 6). **P*<0.05 versus the normal group; #*P*<0.05 versus the HFD group.

Effects of JCM, GG, and their combination on fatty liver induced by HFD

The liver weight and size of mice were markedly increased by the HFD, which were greatly reduced following treatment with all samples to a level similar to that of the normal group (Figure 6A-B) (P>0.05). Moreover, the livers of HFD-fed mice were visually pale, whereas the livers of sample-treated mice were normal (Figure 6B). Histologically, there was a significant increase in lipid accumulation in the liver of the HFD group. However, hepatic lipid accumulation was attenuated by the treatment received by the JCM320, JCM320 + GG150, and GG150 groups.

DISCUSSION

The prevalence of obesity has been increasing, and it is now a global epidemic. Obesity is a potential cause of chronic diseases such as hypertension, type-2 diabetes mellitus, cancer, and cardiovascular diseases. Hence, the prevention and treatment of obesity are critical for health (Caballero, 2007; Stein and Colditz, 2004). Stats showing obesity are quite high, affecting around 1.6 billion people worldwide and is increasing day by day which is an alarming situation for healthcare sector (Rodgers et al., 2012). Although pharmaco-logical drugs such as orlistat have been used to treat obesity, the possibility of adverse side effects should not be ignored (Saltiel, 2016). Accordingly, there have been increased interests in the use of natural products as an anti-obesity dietary supplement, while ethnomedicine and Mediterranean diets have also been known to be effective in treating several ailments including obesity and other related cardiovascular diseases (Kim et al., 2018; Lu et al., 2018; Heber, 2003).

JCM is old rice (*Oryza sativa L.*) used in traditional Korean medicine to treat fever, diarrhea, vomiting, edema, and abdominal disturbance. Our present study was the first to examine the properties of JCM and its synergic effect with GG on the progression of obesity.

The intake of HFD increased the body size, weight gain, and feed efficiency of C57BL/6J mice, which were reduced by all 4 samples. Body weight gain primed by the HFD was effectively reduced in the GG150 group, whereas feed efficiency was not altered in all treated groups. Consistent with the results of body weight change, the accumulation of total WAT was significantly suppressed in the GG150 group. In addition, the serum lipid profile was analyzed, which is closely related to lipid disorders including stroke, hypertension, atherosclerosis, type-2 diabetes and coronary heart disease (Poirier et al., 2005). The serum concentration of glucose was significantly increased in the HFD group and partially reduced in the JCM320 + GG150 and JCM640 + GG150 groups. Dietary cholesterol is directly related to increased serum LDL concentration which can be further linked with atheromas. Clinical evidences shows that lowering LDL cholesterol is effective in minimizing risk factors for atherosclerosis or various cardiovascular conditions (Wang et al., 2011), while several herbal extracts have been known to possess anti-obesity effects and reduce cholesterol levels (Rodgers et al., 2012). Our results represent that LDL was significantly decreased in the JCM640+GG150 and GG150 groups compared with the HFD group. These results suggest that the treatment of JCM640 + GG150 may be the most effective in preventing hyperlipidemia and other lipid-related disorders.

Adipose tissue mass increase is results from two different processes. One is an increase in adipogenesis, and the other is an increase in adipocyte size due to the storage of fat i.e. either due to micro- or macro-vesicular lipid accumulation in the adipocytes (Bays et al., 2008; Kubota et al., 1999). When the progression of adipogenesis is impaired during excessive caloric intake, adipocytes undergo hypertrophy due to excessive energy storage. The hypertrophy of adipocytes leads to metabolic disorders including insulin resistance. In this study, a HFD was found to greatly increase the total fat mass but also induce the hypertrophy of adipocytes in epididymal WAT. The treatment of JCM320 + GG150, JCM640 + GG150, and GG150 suppressed the total WAT weight and adipocyte hypertrophy, with only the GG150 group showing a statistically significant decrease. This result suggests that the partial inhibitory effects of the combination of JCM and GG on the accumulation of adipose tissue may be attributed to the effect of 150 mg/kg GG.

There are two types of adipose tissue depending on the phenotype; WAT and BAT. When caloric intake is more than sufficient, the excess energy is accumulated as triglycerides mainly in the adipocytes of WAT (Shen et al., 2014). In contrast, BAT directly expends energy to generate heat by the oxidation of uncoupled fatty acid via UCP-1, which is expressed on the mitochondrial inner membrane (Tsubone et al., 2005; Spiegelman and Flier, 1996). Therefore, the regulation of body temperature is related to the regulation of body weight. Recently, it has been found that following certain stimuli, oxidative metabolism and UCP-1 gene expression are enhanced in WAT, known as the 'browning' of white adipocytes into beige adipocytes (Nedergaard and Cannon, 2014; Lo and Sun, 2013; Nedergaard and Cannon, 2010). The differentiation of beige adipocytes in WAT might improve metabolic health, and it has been known to possess anti-diabetic and anti-obesity activities in rodent models. PGC-1α activates the transcription of the mouse UCP-1 gene via PPARγ. Therefore, a novel approach in obesity therapeutic treatment may involve the induction of the browning of WAT using certain stimuli including pharmacological drugs such as PPARγ agonists (Wu et al., 2013). Here in current study, we assessed the expression levels of the UCP-1 and PGC-1α gene in mouse epididymal WAT. The BAT from control mice showed a high expression level of both UCP-1 and PGC-1α. However, treatment with all samples did not alter gene expression levels, indicating that both JCM and GG did not induce the browning of WAT, and the reduction in body weight is not mediated by the browning mechanism.

NAFLD is one of the most common complications of obesity which may lead to steatosis and cirrhosis of liver. Such patients are at greater risk and more vulnerable to develop cardiovascular diseases, where ultimately, lowering lipid levels become primary goal to deal with cardiovascular ailments (Loomba and Sanyal, 2013; Wang et al., 2011). Fatty liver and hepatic steatosis result from the accumulation of fatty acids in the liver, which are released from adipose tissue (Lee et al., 2018; Yang et al., 2013). In our study, morphological and histological changes in liver can be seen evidently, indicating that HFD control group mice produced fatty liver by fat accumulation in the liver. The liver size and weight of mice in the HFD group were notably increased and were reduced following treatment with all samples to a level similar to that of the normal group. Moreover, histological images revealed the progression of hepatic steatosis in the liver of the HFD group. Microvascular or macrovesicular lipid accumulation is quite visible in HFD fed mice group which cause hepatic steatosis. It is also evident that size of hepatocytes is increased in HFD control group compared to normal which is also an indication of fat accumulation in hepatocytes, whereas, liver size and hepatocytes size is quite close to normal in

treatment groups. Our results show that liver steatosis is markedly suppressed by treatment with JCM and GG, consistent with the results of serum GPT level. No statistically significant difference was observed in the kidney weights (data not shown) and the levels of serum CRE and BUN among all groups, suggesting that the HFD and our samples did not affect renal function. In conclusion, JCM with GG could exert partial anti-obesity effects by reducing body weight gain, preventing the accumulation of adipose tissue, and suppressing the progression of fatty liver disease. The findings may provide insights into the anti-obesity mechanisms of JCM and GG in the liver.

Acknowledgments

Current research work was funded by the National Research Foundation (NRF), Republic of Korea (2018R1D1A1A09083797).

Authors' contribution

MK, THK and MHR designed and conceptualized the study. MK, YYL and MI performed experiments. MK and YYL wrote the manuscript. SKC, BSJ, JEH, DK, THK and MHR provided technical assistance in experimentation and analyzing the data. THK and MHR supervised the work. All authors have read and approved the final manuscript

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