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Original Article

In vitro and in vivo effects of ethanol extract combined with Curcumae Radix and Glycyrrhizae Radix et Rhizoma on menopausal metabolic disturbances

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Abstract: Curcumae Radix (CR) and Glycyrrhizae Radix et Rhizoma (GR) extracts have been used as health supplements in traditional medicine. This study was performed to evaluate the effects of combined CR and GR extracts (CR+GR) on metabolic complications related menopausal symptoms. We found a significant results that CR+GR extracted using ethanol stimulated the growth of MCF-7 cells in estrogen activity and was attenuated in lipid deposition of HepG2 cells treated with MβCD compared to CR and GR treatments each. To investigate the situation, an experimental menopause rat model with dyslipidemia was induced by surgical bilateral ovariectomy (OVX) and high fat high cholesterol (HFHC) diet in female rats. OVX rats fed HFHC (OVX-HFHC) showed a shift in weight gain, elevated serum cholesterol, altered liver enzymatic parameters and enhanced liver injury compared to the NC and HFHC groups. However, administration of CR+GR, in particular 200 or 450 mg/kg/day, inhibited the increase in body weight gain and lipid metabolic disturbances, lowering total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) compared to the OVX-HFHC group. Furthermore, CR+GR (200 or 450 mg/kg/day) ameliorated the serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine transaminase (ALT) compared to the OVX-HFHC group. Moreover, CR+GR (200 or 450 mg/kg/day) attenuated not only hepatic steatosis but also larger adipocytes. Our study demonstrated that combined treatment with CR and GR attenuated metabolic complications induced by OVX and HFHC diet, suggesting that this effect may regulate and prevent the acceleration of cardiovascular disease (CVD) after menopause.

Keywords: Curcumae Radix (CR), Glycyrrhizae Radix et Rhizoma (GR), menopause, estrogen activity, dyslipidemia, metabolic disturbances

Introduction

Menopause is the stage at which ovarian function is lost and the secretion of estradiol declines. This estrogen deficiency causes a consequence of natural changes from aging or surgically undergoing bilateral oophorectomy [1]. At the onset of menopause, there are significant metabolic alterations such as body composition and a shift toward atherogenic serum lipid levels. In addition, these menopausal symptoms cause development of cardiovascular and hepatic diseases in the long term [2]. Natural menopause typically occurs in a woman's late 40's or early 50's, and life expectancy is gradually increasing; therefore, one third of women's lives is spent in the postmenopausal stage [3]. Thus, new therapeutic agents for climacteric symptoms need to be discovered to improve quality of life in postmenopausal women.

Ovariectomy (OVX) in animals induces estrogen deficiency, causing body weight gain, increased cholesterol levels and progression of hepatic lipid deposition, closely mimicking what is observed in menopausal women. High fat high cholesterol (HFHC) diet aggravates the effect of OVX and leads to profound metabolic changes, such as increased fat accumulation that characterizes obesity, dyslipidemia, steatohepatitis and visceral adiposity showing increased adipocyte size [4, 5].

Curcumae Radix (CR) is a perennial herbaceous plant belonging to the roots of *Curcuma longa*

L. and composed of a rhizome that is used for medicinal purposes. When the root of CR is cut into, it has golden yellow color or orange color. CR contains 0.3% of curcumin as a yellow pigment and 1-5% of turmerone and dehydroturmerone as an essential oil with distinctive fragrance [6]. CR has multiple pharmacological effects including choleretic [7], anti-cancer [8], anti-inflammatory [9] and anti-oxidative effect [10].

Glycyrrhizae Radix et Rhizoma (GR) is a perennial plant belonging to fabaceae, its root is mainly used. It has the advantage of easily mixing with other components as a surfactant, thus it is used in medicines, health foods, and cosmetics [11, 12]. Glycyrrhizae Radix Praeparata is produced by roasting GR until the outer color turns to purple [13]. As the main components of GR, glycyrrhizin, saponin, flavonoid, and polysaccharides such as liqcoumarin, glucose, sucrose, mannitol, and asparagine. Especially, glycyrrhizin which is an important functional material can lower serum cholesterol content [14].

Of importance to the current study associated with menopause, extracts of CR and GR in animal models have demonstrated vascular relaxation [15], osteoblast proliferation [16], antioxidative effect [17] and reducing hot flashes [18] by estrogenic activity. However, little is known about the effects of combined CR and GR extracts on climacteric symptoms in an animal model. In relation to the above, we investigated the effects of combined extract with CR and GR at varying doses on metabolism in OVX rats fed HFHC diet.

Materials and methods

Extracts of herbs

CR and GR were purchased from Daejeon herbal market (Daejeon, Korea), and all herbs samples were authenticated by a morphological expert, Dr. Gi Jung Kil at the Joongbu University. It was extracted with hot water or 70% ethanol at 70°C for 12 h. The extracts were filtered with a 0.45 μm filter, and concentrated by a rotary evaporator. The concentrates were then freeze-dried. The extracts (CR; KIOM 130110, GR; KIOM 130067) were stored at Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea) until used in this experiment. Combined powder of herbs was mixed CR extract powder and GR extract powder, respectively (CR+GR). Combined ratio was 1:1 (CR yield W/GR yield W), was abbreviated as CR+GR. This powder was used as a sample for cell and animal experiment.

Cell culture and treatment

The human breast carcinoma MCF-7 cell line was purchased from the American Type Culture Collection (Rockville, MD) and the human hepatoma HepG2 cell line was purchased from the Korean cell line bank (Seoul, Korea). MCF-7 and HepG2 cells were grown in RPMI/DMEM culture medium (supplemented with 10% FBS and 100 U/mL penicillin-streptomycin, Hyclone) at 5% CO_2 and 37°C. At 80% confluence, cells were harvested using 0.25% trypsin (Hyclone) and were sub-cultured according to the selected experiments. Cells were allowed to attach to the surface for 24 h prior to extracts exposure. MCF-7 cells were seeded at a density of 1×10^4 cells/well in 96 well plates, and triplicates of each cell line were plate. After incubating for 24 hours, the media were treated with PBS, 17*β*-estradiol (Sigma-Aldrich) and CR+GR extracts for 8 h. HepG2 cells were seeded at a density of 1×10^5 cells/well in 24-well plates and incubated in 0.2% BSA-DMEM containing 20 μg/mL methyl-β-cyclodextrin (MβCD, Sigma-Aldrich), along with 30 μM simvastatin or CR+GR extracts for 8 h. The cells were exposed to MβCD mixed with palmitic acid to acutely deplete cellular cholesterol.

Cell proliferation assay

Cell proliferation was determined by Sulforhodamine-B (SRB) assay. After the treatment, cells were fixed with trichloroacetic at 4°C for 1 h. Then the cells were washed and stained for 15 min with 0.4% SRB (dissolved in 1% acetic acid). Subsequently, each well was washed four times with 1% acetic acid, and left to dry at room temperature. The bound protein stain was solubilized in a tris-base (tris (hydroxymethyl) aminomethane) solution. The optical density (OD) was read at 450 nm in a microplate reader.

Oil Red O staining

HepG2 cells were washed with PBS and fixed with 10% formaldehyde for 10 min. After fixa-

tion, cells were washed with PBS and incubated in 60% isopropanol. Cells were dried and stained with Oil Red O solution (stock solution, 3 mg/mL in isopropanol; working solution, 60% Oil Red O stock solution diluted in water) for 10 min at room temperature. Cells were washed four times in water, images were acquired, and then cells were dried. To quantify Oil Red O content levels, isopropanol was added to each sample, after shaking at room temperature for 10 min, followed by absorbance measurement at 500 nm on a spectrophotometer.

Animals and experimental design

The animals used in this study were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Institutional Animal Care and Use Committee (IACUC) of Institute of Oriental Medicine (approval number: 14-017). 42 female Sprague Dawley (SD) rat aged 7 weeks (200-220 g) were provided by Orient Bio (Seongnam, Gyeonggi, Korea) and adopted to a normal diet for 1 week. The rats were maintained at a regular 12 h light/dark cycle, at 22 ± 1°C, with a relative humidity of 50 ± 5%, and were fed a commercial diet (Ralston-Purina, St. Louis, MO, USA). Following 1 week of adaptation, the rats were randomly divided into two groups, the sham operation group and OVX operation group. OVX surgery was performed by ligation and excision of the ovaries along the upper horns, under isoflurane-nitrous oxide anesthesia, through aseptic incisions of the dorsal skin and muscle layers. Sham surgery was underwent the same procedure except for the removal of the ovaries. After a 1-week recover period, rats were randomly subdivided by weight into 7 groups of 6 animals in each group based on the type of diet and treatments : (A) sham operated rats fed normal chow(NC) and administered saline (NC); (B) sham operated rats fed HFHC diet and administered saline (HFHC); (C) OVX operated rats fed HFHC diet and administered saline (OVX-HFHC); (D) OVX operated rats fed HFHC diet and administered simvastatin 20 mg/kg/day (SV 20); (E), (F), (G) OVX operated rats fed HFHC diet and administered CR+GR 50, 200 and 450 mg/kg/day, respectively (CR+GR 50, CR+GR 200 and CR+GR 450). NC diet was a commercial diet (Ralston-Purina, St. Louis, MO, USA) and HFHC diet was western-type diet, which including 45% (w/w) fat and 0.15% (w/w) cholesterol (Feedlab, Gyeonggi-do, Korea). Body weight was recorded once a week through experimental period and the body weight gain was calculated by the equation: final body weight-initial body weight. Food intake was measured 3 times a week. Fresh chow was provided and any remaining chow from the previous day was weighed and discarded.

Blood collection and biochemical analyses

At the end of experiments, rats were anesthetized with Zoletil (1 mL/kg intraperitoneally) and sacrificed after overnight fasting. Blood samples were collected from the inferior vena cava and centrifuged at 3,000 rpm for 10 min to collect the serum. TC, TG, HDL-C, ALT and AST levels in the serum were performed enzymatically using a Roche Modular P Autoanalyzer (Roche Diagnostics, Indianapolis, IN). The concentration of LDL-C was calculated using the Friedewald equation [19]. Atherogenic index were calculated from TC and HDL-C levels [20].

Histological analysis of liver and ovarian adipose tissue

After collecting the blood samples, liver and adipose tissue from ovarian fat pads were immediately excised, weighed and fixed overnight in 10% neutral buffered formalin at 4°C. The collected liver fragments ovarian fat pad sections were stained with hematoxylin and eosin (H&E) according to the standard histological procedures. 3 rats per group were randomly selected. The lipid and nuclei of the liver cells were stained with H&E. A diagnosis of fatty liver was made based on the presence of macro- or micro-vesicular fat in >5% of the hepatocytes in a given slide. Micrographs were taken at 200× magnification. In 3 randomly selected micrographs per section, the diameter of adipocytes was measured using an imaging program (Image J, NIH, USA) and then the number of adipocytes falling into each field with intervals of 5 μm was counted.

Statistical analysis

Values are presented as mean ± SEM. Statistical significance was analyzed by one-way ANOVA, and the Duncan's multiple range test for multiple comparison were used to identify differences among groups; *P* values less than 0.05 were considered significant.

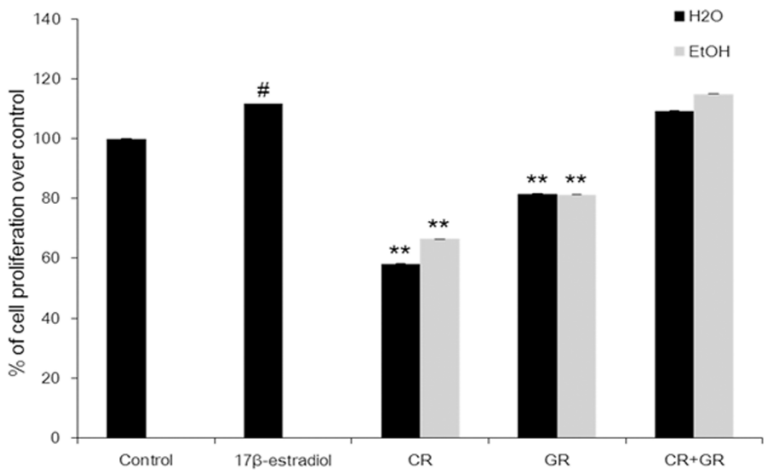

Figure 1. Effect of CR+GR on proliferation in MCF-7 cells. Values are expressed as mean ± SEM. n=3 per group. #p<0.05 vs control group; **p<0.01 vs 17β-estradiol group.

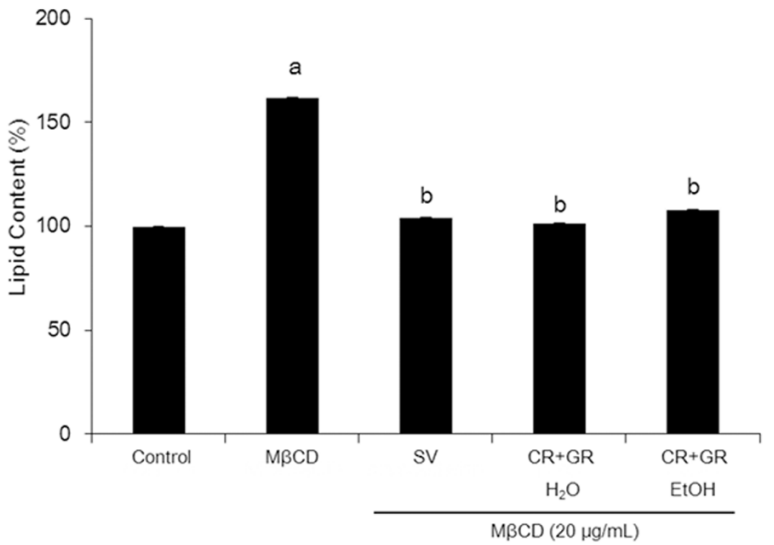

Figure 2. Effect of CR+GR on lipid accumulation in HepG2 cells. Values are expressed as mean ± SEM. n=3 per group. #p<0.05 vs control group; ** p<0.01 vs MβCD group.

Results

Estrogen activity

We evaluated an estrogen activity to determine the effect of CR+GR on MCF-7 cell proliferation. MCF-7 cells were monitored in response to 17β-estradiol (10^{-9} M) and extracts at concentrations starting from 10 μg/mL. Data are expressed as a percentage of the control cell cultures. The CR ethanol extract was observed to cause a decrease (66.4%) in cell growth compared with that of untreated control cells. Although the estrogen activity decreased significantly to 66.4% and 81% when CR and GR ethanol extracts were used individually, CR+GR ethanol extracts showed a tendency to rise (114.96%). The results indicate that CR+GR had higher binding affinities for estrogen receptors in MCF-7 cells. CR+GR ethanol extract may be useful for the treatmentof estrogen-related conditions such as menopause (**Figure 1**).

Lipid accumulation inhibition

We examined the inhibitory effects of CR+GRon lipid accumulation in HepG2 cells incubated with Oil Red O. A significant increase in lipid deposition was observed in HepG2 cells treated with MβCD; however, this effect was attenuated in simvastatin. Furthermore, the intracellular lipid content was significantly reduced following treatment with CR+GR water and ethanol extracts (**Figure 2**).

Body weight, weight gain, food intake and feed efficiency

Saline, simvastatin (positive control) and CR+GR at varying doses were orally administered to sham or OVX rats with NC or HFHC diet for 8 weeks. At the end of the experiment, the OVX-HFHC group gained 30% more body weight compared to the control NC group and 12% more body weight compared to the HFHC group. The SV 20 group administered 20 mg/kg/day (SV 20) gained 10% less body weight compared to the OVX-HFHC group. The groups administered 200 and 450 mg/kg/day (CR+GR 200, CR+GR 450) gained 9% less and 8% less compared to the body weight gain in the OVX-HFHC group, respectively (**Figure 3A**, **3B**). However,

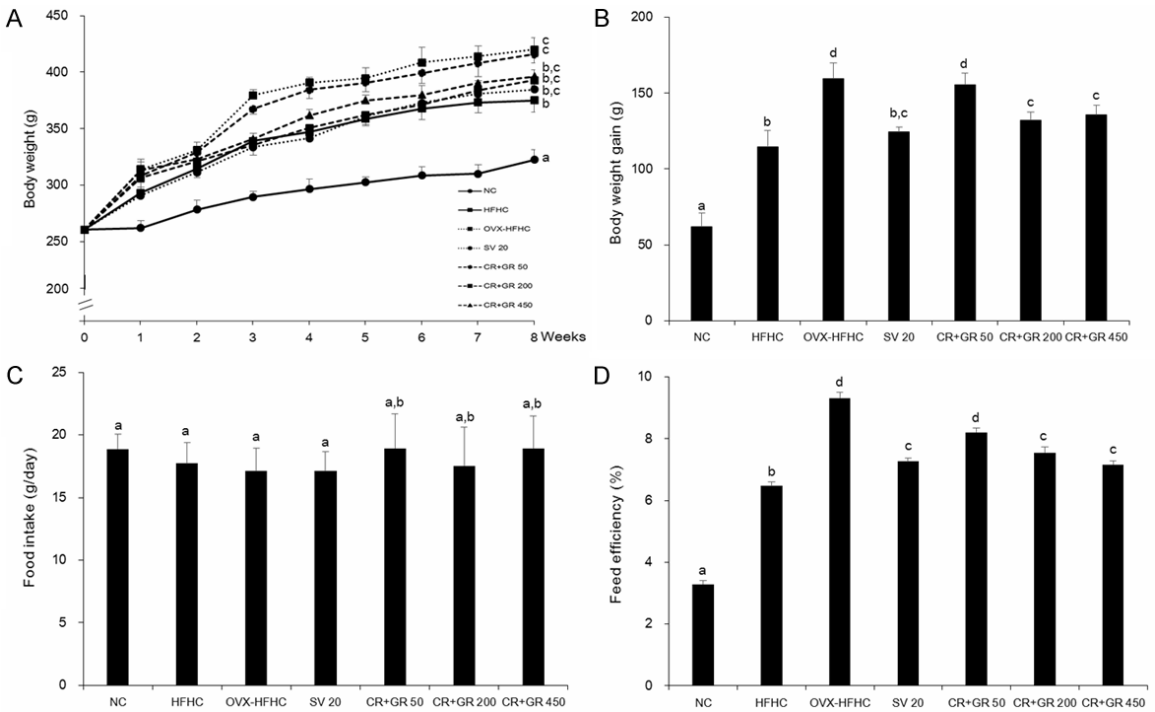

Figure 3. (A) Time course of body weight. [b]$p<0.01$ vs NC group; d $p<0.01$ vs HFHC group; [e]$p<0.05$, [f]$p<0.05$ vs OVX-HFHC group. (B) body weight gain. [b]$p<0.01$ vs NC group; [d]$p<0.01$ vs HFHC group; [f]$p<0.05$ vs OVX-HFHC group. (C) food intake and (D) food efficiency in ovariectomized (OVX) rats administered with CR+GR. [b]$p<0.01$ vs NC group; [d]$p<0.01$ vs HFHC group; [f]$p<0.05$ vs OVX-HFHC group. Values are expressed as mean ± SEM. n=6 per group.

Table 1. Changes of the plasma lipids, atherogenic index (AI) in ovariectomized (OVX) rats treated with CR+GR

Parameters	Treatment groups						
	NC	HFHC	OVX-HFHC	SV 20	CR+GR 50	CR+GR 200	CR+GR 450
Serum lipid values (mg/dL)							
TC	67.40 ± 8.02[b]	99.20 ± 2.77[d]	103.50 ± 3.56[d]	93.00 ± 4.80[c,d]	100.17 ± 7.57[d]	85.17 ± 7.94[c]	88.33 ± 4.37[a]
TG	54.80 ± 6.80[a]	86.40 ± 4.83[b]	98.50 ± 8.76[c]	82.80 ± 5.63[b]	93.17 ± 7.36[b,c]	86.33 ± 2.58[b]	88.33 ± 2.07[b,c]
HDL-C	41.00 ± 2.45[a]	39.80 ± 6.87[a]	34.33 ± 2.80[a]	37.00 ± 2.74[a]	38.83 ± 4.26[a]	41.33 ± 3.72[a]	41.00 ± 3.63[a]
LDL-C	15.44 ± 4.96[a]	42.12 ± 7.52[c,d]	49.47 ± 6.64[d]	39.44 ± 5.00[b,c,d]	42.70 ± 9.31[c,d]	26.57 ± 10.47[a,b]	29.67 ± 4.65[b,c]
AI	0.64 ± 0.12[a]	1.56 ± 0.46[b,c]	2.03 ± 0.29[c]	1.53 ± 0.26[b,c]	1.61 ± 0.38[b,c]	1.08 ± 0.32[a,b]	1.17 ± 0.19[a,b]
Enzymatic parameters (UI/L)							
ALT	30.20 ± 5.72[a]	51.60 ± 4.51[c,d]	57.83 ± 4.26[d]	44.40 ± 6.80[b,c]	43.17 ± 5.88[b,c]	40.50 ± 1.87[b]	42.83 ± 2.64[b,c]
AST	61.80 ± 12.85[a]	124.00 ± 16.05[c,d]	131.20 ± 11.86[d]	95.00 ± 2.16[b]	120.83 ± 10.07[c,d]	110.17 ± 5.53[b,c]	107.67 ± 6.56[b,c]

Values are expressed as mean ± SEM (n=6). [b]$p<0.01$ vs NC group; [c]$p<0.05$ vs HFHC group; [e]$p<0.05$, [f]$p<0.05$ vs OVX-HFHC group. NC, Normal Control, HFHC, High Fat High Cholesterol, OVX, Ovariectomy, SV, Simvastatin, CR, Curcumae Radix, GR, Glycyrrhizae Radix et Rhizoma, TC, Total Cholesterol; TG, Triglyceride; HDL-C, HDL Cholesterol; LDL-C, LDL Cholesterol. AI, Atheroscrelosis Index; (Total Cholesterol-HDL Cholesterol)/HDL Cholesterol. ALT, aspartate aminotransferase; AST, alanine aminotransferase.

the mean daily food intake was slightly higher in the CR+GR administered groups compared to the other experimental groups (**Figure 3C**). The feed efficiency ratio (FER) showed a similar pattern with body weight gain (**Figure 3D**). Based on these findings, oral administration of CR+GR (200 or 450 mg/kg/day) to OVX rats prevented weight gain induced by estrogen deficiency and HFHC diet.

Biochemical analysis

Consumption of the HFHC diet by sham operated rats (HFHC) induced hyperlipidemia by increasing levels of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) relative to those in NC group. TC, TG and LDL-C levels were markedly higher in the OVX-HFHC group compared to the NC group.

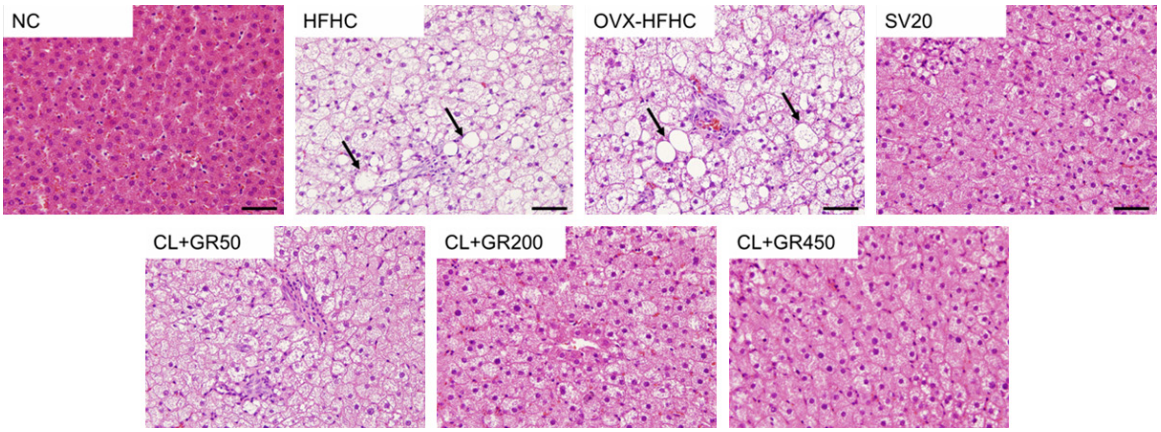

Figure 4. Histological images of liver. Groups: NC, sham-operated rat fed normal chow; HFHC, sham-operated rat fed HFHC diet; OVX-HFHC, ovariectomized rat fed HFHC diet, SV20, ovariectomized rat fed HFHC diet and administered simvastatin 20 mg/kg/day; CR+GR 50, CR+GR 200, CR+GR 450, ovariectomized rat fed HFHC diet and administered CR+GR 50, 200 and 450 mg/kg/day. Magnification: ×200 and scale bars represent 50 μm. The arrows indicate fatty hepatocytes.

However, CR+GR decreased the OVX-HFHC induced serum TC, TG and LDL-C levels. Administration of 20 mg/kg/day of simvastatin (SV 20), a well-known lipid lowering drug, reduced TC, TG and LDL-C levels compared to the OVX-HFHC group. In addition, CR+GR decreased TC, TG and LDL-C levels; although they were still higher than the control NC group, they were lower than the SV 20 group levels. In particular, 200 or 450 mg/kg/day of CR+GR administration (CR+GR 200, CR+GR 450) showed dramatic reductions of TG and LDL-C levels compared to the OVX-HFHC group. TC levels were 22% lower in the CR+GR 200 group and 17% lower in CR+GR 450 group compared to the OVX-HFHC group. LDL-C levels were 86% lower in the CR+GR 200 group and 67% lower in the CR+GR 450 group compared to the OVX-HFHC group. There were no significant changes in HDL-C levels among the experimental groups (**Table 1**). The atherogenic index (AI) was 143% in the HFHC group and 217% in the OVX-HFHC group compared to the NC group. The CR+GR 50 group showed decreased AI and a level similar to that of the SV 20 group used as a positive control. In addition, the CR+GR 200 and CR+GR 450 groups showed 88% and 74% reductions compared to the OVX-HFHC group, respectively (**Table 1**). Serum cholesterol levels among the groups significantly correlated with serum ALT and AST levels; therefore, we evaluated the impact of CR+GR on enzymatic profiles including ALT and AST levels. The OVX-HFHC group showed dramatic elevations of ALT and AST, serum parameters of liver injury, compared to the NC group. The SV 20 group showed 30% lower ALT and 38% lower AST compared to the OVX-HFHC group. In addition, administration of CR+GR caused 34%, 43% and 35% reductions of ALT levels and 9%, 19% and 22% reductions in AST levels compared to the OVX-HFHC group, significantly. These results indicate that CR+GR is not hepatotoxic to rats at the concentrations used in the present study (**Table 1**).

Morphological changes in hepatocytes

We determined the hepatoprotective effect of CR+GR through analysis of hepatic fat accumulation by hematoxylin and eosin (H&E) staining of liver tissue. Fat accumulation is indicated by the arrowhead. Histological examination of liver from the NC group as a control indicated intact cell architecture, whereas the HFHC and OVX-HFHC groups exhibited hepatic lipid accumulation in the form of large fat droplets present in liver tissue. The CR+GR administered groups presented considerably lower hepatic microvascular steatosis than the HFHC and OVX-c groups. These results showed that CR+GR (200 or 450 mg/kg/day) efficiently inhibited fat accumulation in the liver (**Figure 4**).

Morphological changes in ovarian adipocytes

Figure 5 shows that microscopic ovarian adipose and the size of adipocytes. Histologically, ovarian adipocytes were more irregular in the HFHC and OVX-HFHC groups compared to the

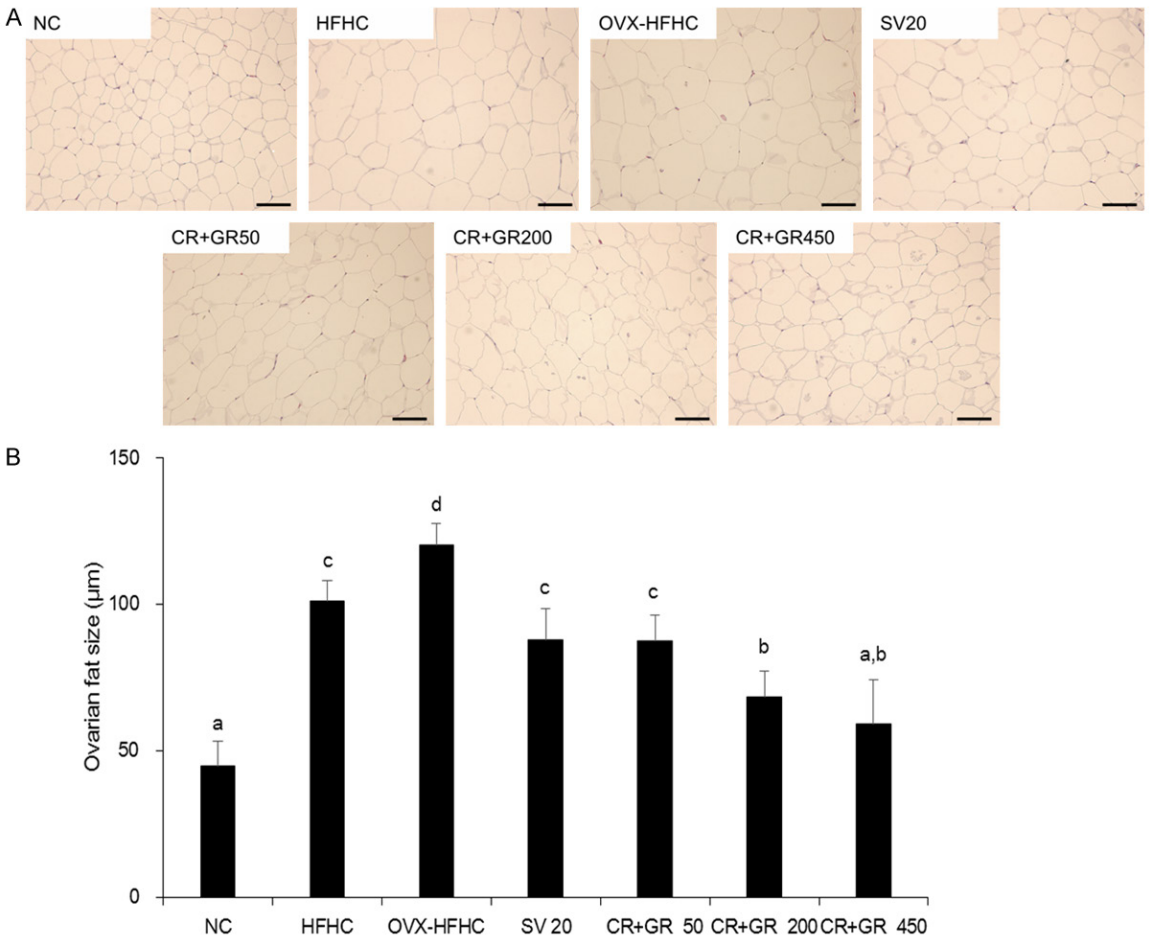

Figure 5. A. Histological images of ovarian fat pad. Groups: NC, sham-operated rat fed normal chow; HFHC, sham-operated rat fed HFHC diet; OVX-HFHC, ovariectomized rat fed HFHC diet, SV20, ovariectomized rat fed HFHC diet and administered simvastatin 20 mg/kg/day; CR+GR 50, CR+GR 200, CR+GR 450, ovariectomized rat fed HFHC diet and administered CR+GR 50, 200 and 450 mg/kg/day. Magnification: ×200 and scale bars represent 50 μm. B. Adipocyte mean diameter. The diameter of adipocytes was measured using Image J software, and then the number of adipocytes falling into each field with intervals of 5 μm was counted. Values are expressed as mean ± SEM. n=3 per group. $^{b}p<0.01$ vs NC group; $^{c}p<0.05$ vs HFHC group; $^{e}p<0.05$, $^{f}p<0.05$ vs OVX-HFHC group.

NC group. Furthermore, ovarian adipose cell diameter in the HFHC and OVX-HFHC groups increased to approximately 105% and 115% compared to the NC group, respectively. However, the CR+GR administered groups showed dose-dependent reductions of ovarian adipose cell size. These results showed that CR+GR (200 or 450 mg/kg/day) efficiently inhibited fat accumulation in ovarian adipocyte tissue (**Figure 5**).

Discussion

Postmenopausal women are more susceptible to cardiac disease due to marked decreases in estrogen levels during the natural atrophy of the ovaries. High-energy diets are widely used in nutritional experiments as a strategy to induce overweight conditions and fat deposition in animals; however, OVX animals fed HFHC diet show greater adipose fat pads and larger adipocytes than animals fed HFHC diet only. Accordingly, OVX is associated with the emergence of various complications such as increased abdominal adiposity and a shift toward a more atherogenic lipid profile. Therefore, estrogen deficiency predisposes postmenopausal women to a 60% greater risk of developing cardiovascular disease (CVD) than prior to menopause [21, 22].

In general, a substance with estrogen-like activity that exists in the plant is called phytoestrogen. Research on the possibility that phytoestrogen has alternative mechanisms of action of estrogen in postmenopausal women are continually reporting. The evaluation of estrogenic activity was carried out in order to find out the efficacy of medicine that could be alternative to estrogen by using the mixture of medicinal herbs such as CR that has alternative mechanisms of action of estrogen in postmenopausal women and antioxidant properties to be of great benefit in women experiencing menopausal symptoms and GR that can be alternative to estrogen. HPLC analysis showed that the index component content was higher in ethanol extract than water extract of CR (not present). MCF-7 cell showed that higher estrogenic activity was shown in the combined extract than ethanol extract of each CR and GR (**Figure 1**). Also, our results in vitro indicate that CR+GR synergistically attenuated impairments of lipid accumulation in HepG2 cells (**Figure 2**). The intracellular lipid content was significantly reduced following treatment with CR+GR in HepG2 cells. Consequently, CR+GR ameliorated increased negative lipid levels and inhibited fat deposition in liver and adipocytes in OVX rats fed HFHC diet.

Our data suggest the potential use of CR+GR (200 or 450 mg/kg/day) to reinforce decreased body weight gain associated with hormone deficiency in female rats (**Figure 3**). The weight gain of OVX rats fed HFHC diet exceeded that of HFHC group with a 44% higher FER. A previous study reported that the FER of OVX rats was far higher than that of normal female rats, suggesting that estrogen deficiency is involved in weight gain and energy metabolism [24]. OVX in rats causes an increase in body weight and food efficiency with elevated plasma leptin, a hormone that helps to regulate energy balance, whereas 17β-estradiol treatment reverses weight gain and food efficiency without changing plasma leptin [25]. Interestingly, considering our result that the FER of the CR+GR treated groups slightly exceeded their increased food consumption, CR+GR suppressed weight gain and food efficiency in OVX rats. These anti-obesity effects of CR+GR may be associated with its action controlling fat synthesis and utilization.

Consistent with the decreases in body weight gain, visceral fat including retroperitoneal and ovarian fat tissues were dissected and weighed (not presented). The retroperitoneal and ovarian fat mass were dose dependently lower in the CR+GR administered groups than in the other groups; therefore, the decrease of body weight gain was accompanied by a depletion of body fat stores. As menopausal symptoms, increased weight gain and abdominal fat have positive associations with dyslipidemia characterized by elevated TC, TG and LDL-C and decreased HDL-C concentrations [26]. The CR+GR (200 or 450 mg/kg/day) significantly lowered plasma TC, TG and LDL-C levels in OVX rats fed HFHC diet inducing altered lipid profile levels.

A series of *in vitro* and *in vivo* experiments in rodents has led to a better understanding that the hypercholesterolemic action in plasma due to increased uptake of dietary exogenous cholesterol, subsequent deposition and inhibited cholesterol absorption as evidence for a reduction in bile acid production and turnover to bile acids. However, high HDL-C is helpful in transporting excess cholesterol to the liver for excretion in the bile [27]. In the present study, although not significantly different, HDL-C levels showed a tendency toward increasing in the CR+GR treated groups (200 or 450 mg/kg/day).

Another noteworthy feature of this study is that the effects of CR+GR (200 or 450 mg/kg/day) treatment on the AI showed remarkable reduction. The AI of plasma, defined as the ratio of plasma concentration of TG to HDL-C, has recently been proposed as a predictive marker for plasma atherogenicity and is positively correlated with cardiovascular disease risk [28]. In total, CR+GR has therapeutic potential in the management and prevention of obesity, hypercholesterolemia and atherogenic diseases for menopausal women (**Table 1**).

In all species that have been studied, the liver plays the central role in maintaining cholesterol balance and regulating the level of circulating lipoprotein cholesterol. It was also reported that dietary cholesterol feeding is characterized by elevated cholesterol synthesis and uptake from circulating lipoproteins and by reduced cholesterol excretion [29]. In addition, It leads to persistent liver injury, resulting in

hepatic inflammation after accumulating inflammatory macrophages in rodents and humans produce various inflammatory cytokines such as TNF-α and IFN-γ. Moreover, the accelerated progression of hepatic steatosis in postmenopausal women as well as experimental research in OVX animals has demonstrated that estrogen deficiency worsens hepatic steatosis [30-32]. Moreover, in the present study, histopathological examination supported that CR+GR (200 or 450 mg/kg/day) significantly reduced the accumulation of fat droplets in hepatic tissues compared with fatty liver formation induced by OVX and HFHC (**Figure 4**). Hepatic steatosis induced by OVX and HFHC diet has a highly significant association with endothelial dysfunctions; therefore, the hepatoprotective effect of CR+GR may be related to enhanced nitric oxide (NO) availability in the vascular system [33]. Therefore, we need to further study whether CR+GR (200 or 450 mg/kg/day) inhibits the spontaneous secretion of inflammatory cytokines in the liver or stimulates NO production.

Altogether, most previous authors have reported increased visceral adipocyte size and TC levels under induction of hypercholesterolemia by the addition of dietary cholesterol. In addition, adipose tissue secretes several adipokines, such as leptin, adiponectin, TNF-α, and IL-6, which are involved in lipid metabolism and inflammation [34]. The fat-enriched diet greatly altered ovarian adipocyte morphology and size in HFHC and OVX-HFHC groups. However, CR+GR (200 or 450 mg/kg/day) reduced large ovarian adipocytes compared with OVX rats fed HFHC diet, resulting in greater ovarian adipocyte hypertrophy. Additionally, it would be important to demonstrate an association between adipokines and inflammatory markers during administration of CR+GR. Collectively, these studies indicate the potential of water extracts of CR and GRin future strategies toward drug development relieving menopause symptoms related to metabolic abnormalities.

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Disclosure of conflict of interest

None.

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