Jueming prescription and its ingredients, semen cassiae and Rhizoma Curcumae Longae, stimulate lipolysis and enhance the phosphorylation of hormone-sensitive lipase in cultured rat white adipose tissue

YUE ZHANG[1], JIAOJIAO LI[2] and XIUYING WEN[3]

[1]Department of Endocrinology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430077; [2]Department of Pediatrics, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; [3]Department of Traditional Chinese Medicine and Endocrinology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430077, P.R. China

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Abstract. The present study aimed to investigate the effect of jueming prescription (JMP) and its ingredients, semen cassiae (SC) and Rhizoma Curcumae Longae (RCL), on lipolysis, and to examine their effect on the phosphorylation of hormone-sensitive lipase (HSL) in cultured rat white adipose tissue (WAT). Retroperitoneal WAT was aseptically excised from adult male Sprague-Dawley rats, minced into uniform sections and subjected to *ex vivo* culture for 24 h. The tissue sections were then distributed into a 24-well culture plate and treated with normal saline (vehicle), isoproterenol (ISO), JMP, SC and RCL. Non-esterified fatty acid (NEFA) and glycerol release from the intact WAT explants were determined as a measurement of lipolysis, which were measured using NEFA and glycerol assay kits. The phosphorylation of HSL at Ser563 (P-HSL S563) and 660 residues (P-HSL S660) were determined using western blot analysis. The size of the adipocytes was visualized using hematoxylin and eosin (H&E) staining. It was found that JMP-, SC- and RCL-stimulated lipolysis was responsible for increasing the release of NEFAs and glycerol from the intact WAT *in vitro*. In addition, JMP, SC and RCL increased the levels of P-HSL Ser563: JMP water (JW) extract, 3.52-fold; JMP ethanol (JE) extract, 3.38-fold; SC water (SW) extract, 4.60-fold; SC ethanol (SE) extract, 4.20-fold; RCL water (RW) extract, 6.98-fold; RCL ethanol (RE) extract, 6.60-fold. JMP, SC and RCL also increased the levels of P-HSL Ser660: JW extract, 3.16-fold; JE extract, 2.92-fold; SW extract, 4.57-fold; SE extract, 4.13-fold; RW extract, 5.41-fold; RE 4.96-fold) in the WAT. The RW extract had the most marked effect. The HE staining revealed that JMP, SC and RCL reduced the size of adipocytes in the WAT. In conclusion, JMP and its ingredients, SC and RC, stimulated lipolysis and reduced the size of adipocytes, possibly via the phosphorylation of HSL in cultured rat WAT.

Correspondence to: Dr Xiuying Wen, Department of Traditional Chinese Medicine and Endocrinology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, 39 Lake Avenue, Wuhan, Hubei 430077, P.R. China
E-mail: wenxyoung@hust.edu.cn

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Introduction

Obesity is a medical condition characterized by the expansion of adipose tissue, which is induced by the accumulation of triglycerides (TGs) in adipocytes (1). The adipocytes of white adipose tissue (WAT) are a site of fat storage, as lipid droplets and the balance of lipids stored within the lipid droplets are controlled by TG synthesis (lipogenesis) and degradation (lipolysis). Lipolysis is a biochemical process involving the breakdown of TG stored in lipid droplets, resulting in the release of non-esterified fatty acid (NEFA) and glycerol, and is one of the key processes involved in achieving weight loss (2-5). Therefore, a coordinated stimulation of lipolysis is beneficial in reducing adipose mass and preventing obesity.

Lipolysis, the catabolism of TG in WAT, is regulated by complex regulatory mechanisms. The most well known mechanism involved in mediating lipolysis is the cAMP-dependent protein kinase A (PKA) pathway (6,7). It is well-established that the PKA-mediated phosphorylation of hormone-sensitive lipase (HSL) and perilipin A are critical for stimulating lipolysis, which leads to the hydrolysis of TG and the subsequent release of NEFA and glycerol (7-12). The phosphorylation of HSL protein activates and translocates the enzyme to the surface of lipid droplets, where it hydrolyzes TG (13-17). The Ser563 and Ser660 sites of HSL are the major sites of phosphorylation associated with HSL activity (14,18). HSL is phosphorylated by PKA at Ser563 and Ser660, which activates HSL, increases the translocation of HSL to lipid droplets and enhances lipolysis (19,20). A previous study reported that the phosphorylation of HSL at

Ser563 and Ser660 are reduced in the WAT of obese rats, and the basal phosphorylation of HSL at Ser563 and Ser660 is low in primary adipocytes from obese rats, compared with those from control rats (21). The phosphorylation of HSL at Ser563 and Ser660, and lipolysis are markedly increased upon isoproterenol (ISO; β-adrenergic agonist) treatment in cultured WAT explants from mice (20). ISO also promotes the phosphorylation of HSL at Ser563 and Ser660, and increases lipolysis in primary adipocytes from obese rats and non-obese rats (21,22).

Chinese herbal medicines are important in the treatment of obesity. Increasing evidence from clinical studies and animal experiments study that various Chinese herbal medicines significantly promote lipolysis and the release of NEFA and glycerol in adipocytes and WAT, suppressing adipose tissue weight and body weight gain in humans and animals (2,6,23-26).

In Chinese medicine, it is believed that obesity is associated with the endogenous wet-heat and blood stasis due to the over-consumption of fatty food (27-30). Semen cassiae (SC) has the capacity to clear heat and moisten the intestines, and Rhizoma Curcumae Longae (RCL) can promote qi to activate blood and remove blood stasis (27,28,31). Studies have reported that SC and RCL extracts are effective in enhancing lipolysis, and in reducing the size of adipocytes and body weight in obese rats (2,3,6,32). In our previous study, the Chinese herbal medicine jueming prescription (JMP) was formulated, which consists of SC and RCL (27). Our previous studies showed that JMP reduced body weight, visceral fat weight and adipocyte size in WAT, and also alleviated liver steatosis in rats with diet-induced obesity. It was also shown to reduce rat body weights and to improve lipid metabolism in obese patients (23,27,28). However, there have been no studies analyzing and comparing JMP and its ingredients, SC and RCL, on lipolysis in cultured rat WAT.

The present study investigated the effects of JMP and its ingredients, SC and RCL, on stimulating lipolysis, and whether they are responsible for increasing the release of NEFA and glycerol, and reducing the size of adipocytes and phosphorylation levels of HSL in rat WAT. This was performed using an *ex-vivo* WAT explant culture method.

Materials and methods

Preparation of JMP, SC and RCL. JMP is composed of SC, which is produced in Xianqiao, Zhejiang and RCL, which is produced in Qichun, Hubei, in a 1.5:1 (w/w) ratio. SC and RCL were purchased from Tianji Chinese Herbal Medicine Co., Ltd. (Wuhan, China). All herbal drugs were soaked in eight volumes of distilled water (1:8, w/v) for 12 h at room temperature, followed by boiling three times (2 h each). The JMP water (JW) extract was filtered and concentrated to a concentration equivalent to 0.2 g raw herbs/1 ml water extract (23). The JMP ethanol (JE) extract was concentrated and precipitated with 60% ethanol for 12 h. The solution was evaporated under reduced pressure (~0.1 MPa) to produce a concentration equivalent to 0.2 g raw herbs in 1 ml ethanol extract (33). The SC (SW) extract (0.3 g/ml) and SC ethanol (SE) extract (0.3 g/ml), RCL water (RW) extract (0.4 g/ml) and RCL ethanol (RE) extract (0.4 g/ml) were prepared using the same method used for the JW extract and JE extract. The extracts were stored at 4˚C until they were used.

Chemicals and reagents. M199 medium was from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA), bovine serum albumin (BSA) and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES) were from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). The glycerol assay kit and NEFA assay kit were from Applygen Technologies Inc. (Beijing, China). ISO was from Hefeng Pharmaceutical Co., Ltd. (Shanghai, China), 100 μg/ml streptomycin and 100 U/ml penicillin were from Beyotime Institute of Biotechnology (Shanghai, China), TRIzol reagent and the reverse transcription kit were from Takara Bio, Inc. (Tokyo, Japan), primers were from Wuhan Qingke Innovative Biotechnology Co., Ltd. (Wuhan, China), NP-40 lysis buffer and the BCA protein assay kit were from Beyotime Institute of Biotechnology. Rabbit anti-P-HSL ser563 and rabbit anti-P-HSL ser660 (cat. nos. 4139 and 4126, respectively) were from Cell Signaling Technology Inc. (Danvers, MA, USA), rabbit anti-β-actin (cat. no. TDY051) was from Beijing TDY Biotech Co., Ltd. (Beijing, China).

WAT collection and culture. Adult male Sprague-Dawley rats are purchased from the Experimental Animal Center of Wuhan University (Wuhan, China). All animal experiments were performed in compliance with the Principles for Care and Use of Laboratory Animals approved by the Laboratory of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The animals were housed in a room with temperature controlled at 20±5˚C and 55±5% humidity, under a 12-h light-dark cycle. The rats are fed with standard rat chow (23). Rats with a weight of 300±30 g were used during the experiments. The rats were weighed and anesthetized, and the retroperitoneal WAT was rapidly excised in an aseptic laparotomy procedure (34). The explants of WAT were immediately placed in a culture dish containing pre-warmed (37˚C) phosphate-buffered saline (PBS) solution, and washed briefly to dispel blood. The explants were transferred, discarding the tissue residue and fat droplets, into preheated PBS in another culture dish with. A surgical scalpel was used to cut the explants into uniform sections of ~100±5 mg. The sections were gently washed with PBS, resuspended in PBS, and left standing for 5 min to allow the sections rich in adipocytes to float. The floating sections were collected and transferred into a culture dish containing 25 ml of serum-free M199 with 25 mM HEPES and antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin), and cultured at 37˚C with an atmosphere of 95% air and 5% CO_2 for 24 h. Studies have shown that live adipocytes can survive in fresh adipose tissue and in adipose tissue cultivated for 24 h, with survival rates of 94% each and 87%, respectively (35). At the end of the 24-h incubation period, the dish was removed from the incubator, and pre-warmed PBS was used to clean the adipose tissue, using a sterile gauze to dry the tissues. The tissue sections were distributed into a 24-well culture plate (100 mg tissue/well). The wells are then filled with 1 ml Krebs-Ringer buffer containing 25 mM HEPES, 5.5 mM glucose, and 2% (w/v) BSA. Immediately, the cultures are treated with normal saline (vehicle), 750 nM ISO [which is known to stimulate the release of NEFA in rat WAT through activation of the β-adrenergic receptors (36)] as a positive control, or different concentrations of JW extract, JE extract, SW extract, SE extract, RW extract and RW extract. The

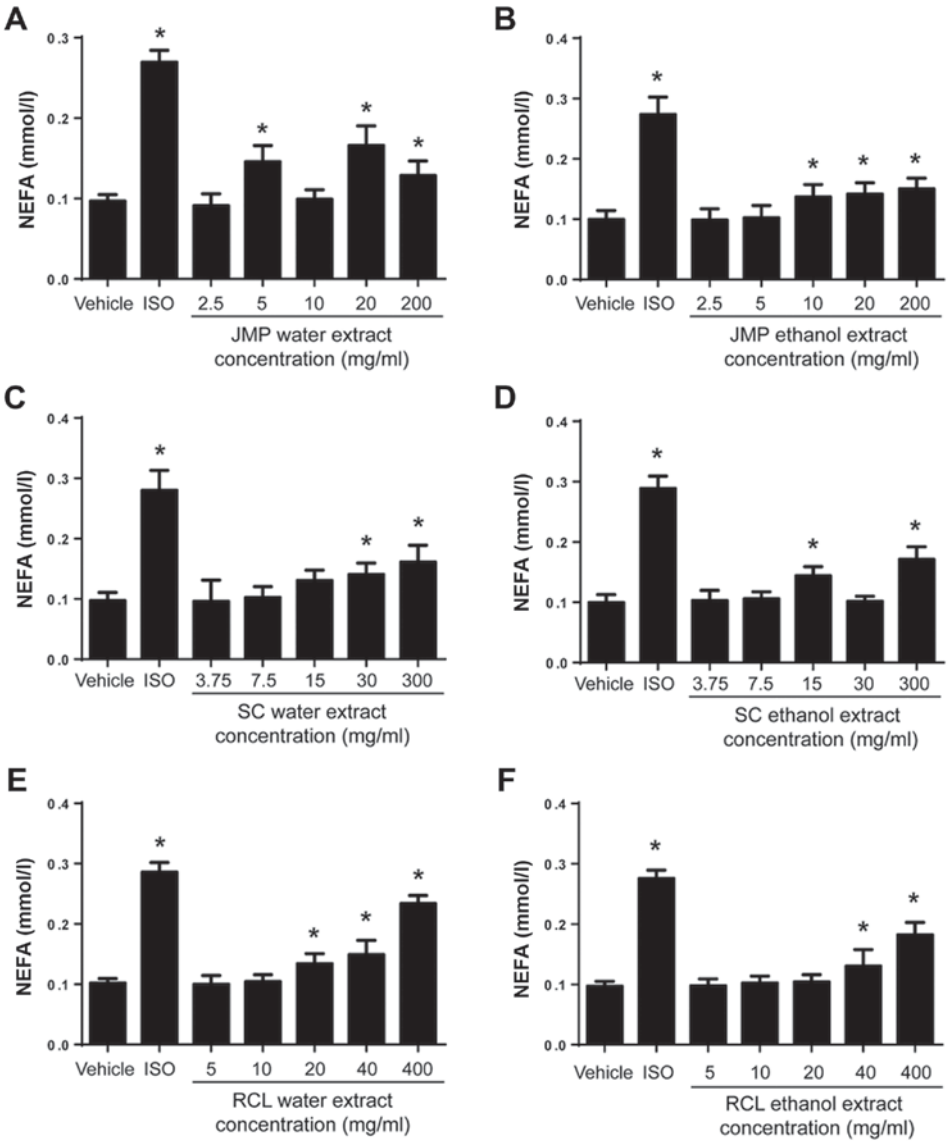

Figure 1. Stimulatory effects of JMP, SC and RCL on the release of NEFA in cultured rat WAT. NEFA release was measured following stimulation with ISO (750 nmol/l) and the indicated concentration of (A) JMP water extract (2.5-200 mg/ml), (B) JMP ethanol extract (2.5-200 mg/ml), (C) SC water extract (3.75-300 mg/ml), (D) SC ethanol extract (3.75-300 mg/ml), (E) RCL water extract (5-400 mg/ml) and (F) CL ethanol extract (5-400 mg/ml) in cultured rat WAT for 90 min. Data are expressed as the mean ± standard error of the mean of at least three independent experiments, each performed in triplicate (n=3). *P<0.05 vs. vehicle. WAT, white adipose tissue; NEFA, non-esterified fatty acid; vehicle, normal saline; ISO, isoproterenol; JMP, jueming prescription; SC, semen cassia; RCL, Rhizoma Curcumae Longae.

cultures were incubated at 37˚C in 5% CO_2 and 95% humidity for 90 min. At the end of the incubation period, the conditioned media were collected from each well. The NEFA and glycerol content were measured using NEFA and glycerol assay kits.

NEFA and glycerol assessment as measures of lipolysis. The assessment of lipolysis was measured by NEFA and glycerol release from WAT into the incubation media. The NEFA and glycerol concentrations in the conditioned media from all samples were determined according to the manufacturer's instructions of the commercial NEFA and glycerol assay kits. The results are expressed as mmol/l of NEFA and mmol/l of glycerol released per 100 mg WAT.

Western blot analysis. The expression of proteins was determined using western blot analysis. The explants of WAT were collected and washed in PBS. The total cellular protein was extracted using NP-40 lysis buffer containing phenylmethanesulphonyl fluoride. The protein concentrations were measured using a BCA protein assay kit according to the manufacturer's instructions. Equal quantities (40 μg) of protein samples were separated by 10 or 12% SDS-PAGE electrophoresis, and then transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5.0% fat-free dry milk in Tris-buffered saline and Tween-20 (TBST) at room temperature for 2 h and incubated with rabbit anti-P-HSL Ser563 (1:1,000), rabbit anti-P-HSL Ser660 (1:1,000), and rabbit anti-β-actin (1:2,000) at 4˚C overnight. Following washing with TBST four times, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies (cat. no. 074-1506, 1:3,000; KPL, Inc., Gaithersburg, MD, USA) for 1 h at room temperature on a rocker with gentle agitation.

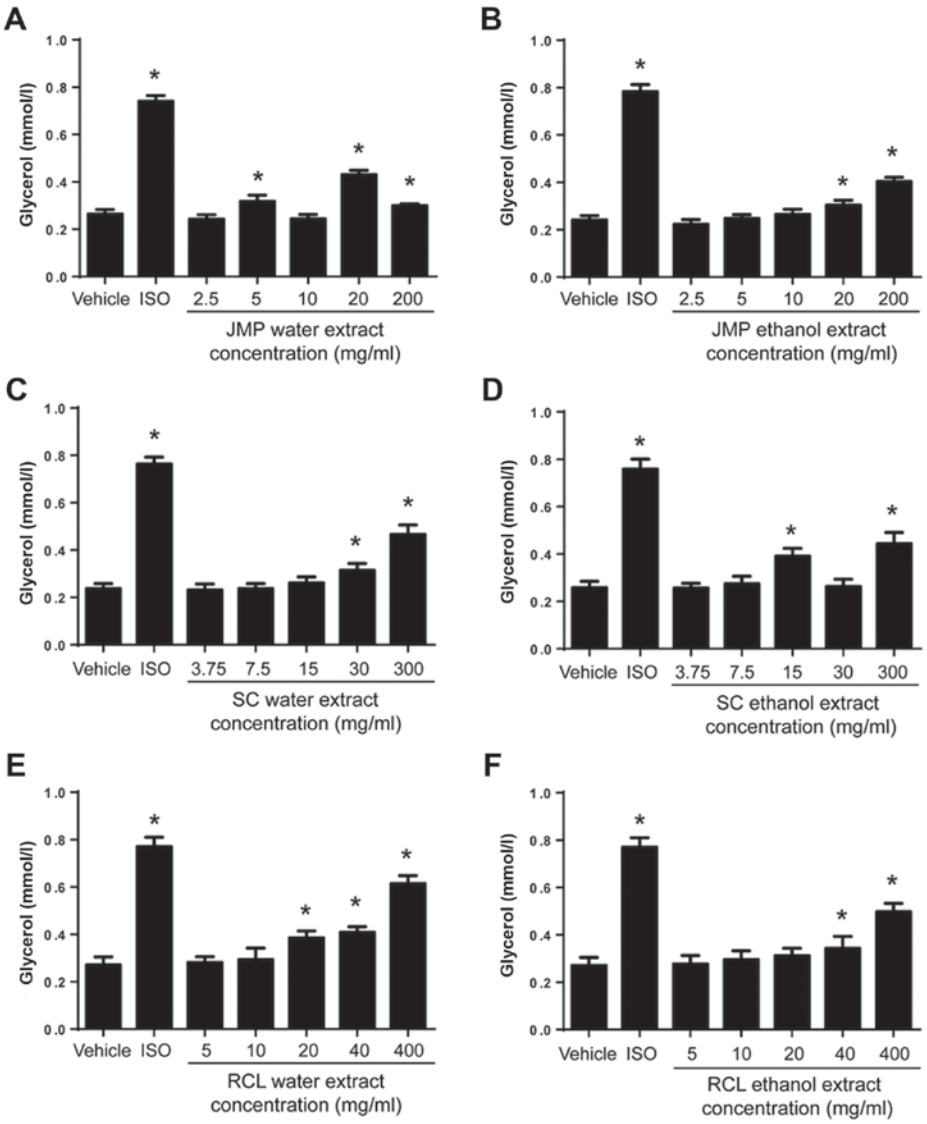

Figure 2. Stimulatory effects of JMP, SC and RCL on glycerol release in cultured rat WAT. Glycerol release was measured following stimulation with ISO (750 nmol/l) and the indicated concentration of (A) JMP water extract (2.5-200 mg/ml), (B) JMP ethanol extract (2.5-200 mg/ml), (C) SC water extract (3.75-300 mg/ml), (D) SC ethanol extract (3.75-300 mg/ml), (E) RCL water extract (5-400 mg/ml) and (F) CL ethanol extract (5-400 mg/ml) in cultured rat WAT for 90 min. Data are expressed as the mean ± standard error of the mean of at least three independent experiments, each performed in triplicate (n=3). *P<0.05 vs. vehicle. WAT, white adipose tissue; vehicle, normal saline; ISO, isoproterenol; JMP, jueming prescription; SC, semen cassia; RCL, Rhizoma Curcumae Longae.

The bands were visualized using ECL reagent, and the band intensities were quantified by densitometry using ImageJ software version 2.0 (National Institutes of Health, Bethesda, MD, USA). The results are presented as the ratio of the optical density of the target bands to β-actin.

Histological analysis. The explants of WAT treated with different concentrations of JCM, SC, RLC were collected, and sections of each explant were fixed in 4% paraformaldehyde for further embedding in paraffin wax. The tissues blocks were further processed using a routine procedure for hematoxylin and eosin (H&E) staining. The stained adipose tissue sections were observed and images were captured under an optical microscope (magnification, x400).

Statistical analysis. All experiment data are presented as the mean ± standard error of the mean of three or more independent experiments. Statistical significance was determined using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Stimulatory effects of JMP, SC and RCL on the release of NEFA in cultured rat WAT. To determine whether JMP and its ingredients, SC and RCL, can stimulate lipolysis in rat WAT, the present study investigated the release of NEFA from JMP-, SC- and RCL-treated rat WAT *in vitro*. The results revealed that 750 nmol/l ISO significantly increased the release of NEFA, compared with vehicle (Fig. 1). Furthermore, the rat WAT was treated with JW extract at various concentrations (2.5, 5.0, 10, 20 and 200 mg/ml). Compared with the vehicle, the release of NEFA was significantly increased in WAT in the rats treated with JMP concentrations of 5.0, 20 and 200 mg/ml, and the

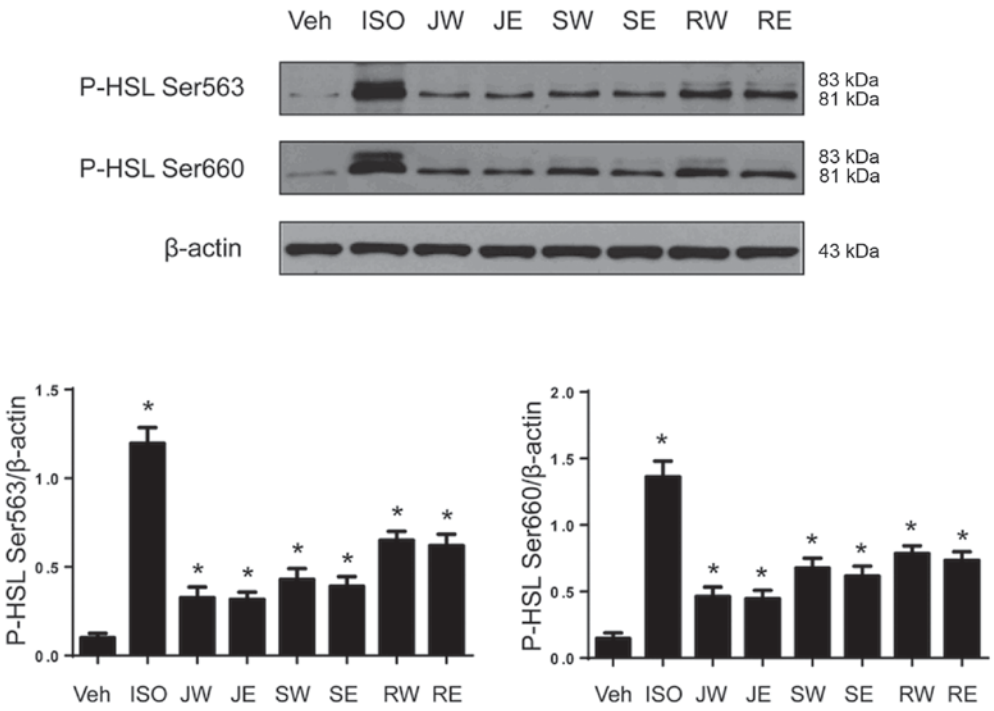

Figure 3. JMP, SC and RCL upregulate p-HSL Ser563 and p-HSL Ser660 in cultured rat WAT. Levels P-HSL Ser563 and P-HSL Ser660 following treatment with JW, JE, SW, SE, RW and RE for 90 min. *P<0.05 vs. Veh. WAT, white adipose tissue; Veh, vehicle, normal saline; ISO, isoproterenol; JMP, jueming prescription; SC, semen cassiae; RCL, Rhizoma Curcumae Longae; P-HSL, phosphorylated of hormone-sensitive lipase; JW, 20 mg/ml JMP water extract; JE, 200 mg/ml JMP ethanol extract; SW, 300 mg/ml SC water extract; SE, 300 mg/ml SC ethanol extract; RW, 400 mg/ml RCL water extract; RE, 400 mg/ml RCL ethanol extract.

maximum effect was observed at a dose of 20 mg/ml (Fig. 1A). JE extract stimulated the release of NEFA at the concentrations of 10, 20 and 200 mg/ml in a dose-dependent manner, compared with the vehicle-treated group, with the maximum effect at 200 mg/ml (Fig. 1B). Treatment with 30 and 300 mg/ml SW extract stimulated the release of NEFA in a dose-dependent manner, with the maximum effect at 300 mg/ml, compared with the vehicle-treated group (Fig. 1C). Treatment with 15 and 300 mg/ml SE extract stimulated the release of NEFA, with the maximum effect at a dose of 300 mg/ml, however this effect was not dose-dependent manner (Fig. 1D). Treatment with 20, 40 and 400 mg/ml RW extract, and 40 and 400 mg/ml RE extract stimulated the release of NEFA in a dose-dependent manner. The maximum effects of RW extract and RE extract were observed at 400 mg/ml (Fig. 1E and F).

Stimulatory effects of JMP, SC and RCL on glycerol release in cultured rat WAT. The present study also determined the release of glycerol from the rat WAT treated with ISO, JMP and its ingredients, SC and RCL. Consistent with the release of NEFA, the effects on lipolysis by the treatments on glycerol were similar in the medium from the cultured rat WAT. Treatment with 750 nmol/l ISO, 5.0, 20 and 200 mg/ml JW extract (maximum at 20 mg/ml), 20 and 200 mg/ml JE extract (dose-dependent with maximum at 200 mg/ml), 30 and 300 mg/ml SW extract (dose-dependent with the maximum at 300 mg/ml), 15 and 300 mg/ml SE extract (maximum at 300 mg/ml), 20, 40 and 400 mg/ml RW extract (dose-dependent, maximum at 400 mg/ml), 40 and 400 mg/ml RE extract (dose-dependent, maximum at 400 mg/ml) stimulated the release of glycerol, compared with release in the vehicle-treated group (Fig. 2A-F).

JMP, SC and RCL upregulate the phosphorylation of HSL at Ser563 and Ser660 in cultured rat WAT. The HSL at Ser563 and Ser660 are major phosphorylation sites associated with HSL activity, and are well known markers of lipolysis (14,18-22). ISO is known to phosphorylate HSL at Ser563 and Ser660 sites, and results in increasing lipolysis, which is used as a positive control (21,22). According to the results of the release of NEFA and glycerol (Figs. 1 and 2), the present study examined the phosphorylation levels of HSL at Ser563 and Ser660 in cultured rat WAT treated with 20 mg/ml JW extract, 200 mg/ml JE extract, 300 mg/ml SW extract, 300 mg/ml SE extract, 400 mg/ml RW extract and 400 mg/ml RE extract. As shown in Fig. 3, consistent with the release of NEFA and glycerol, 750 nmol/l ISO, JW extract, JE extract, SW extract, SE extract, RW extract and RE extract significantly upregulated the phosphorylation levels of HSL at Ser563 (P-HSL Ser563) and Ser660 (P-HSL Ser660), compared with that in the vehicle-treated group. This suggested that JMP, SC and RLC significantly upregulated the expression of P-HSL Ser563 and P-HSL Ser660 in the stimulation of lipolysis in rat WAT *in vitro*. RCL had the most marked effect on increasing the levels of P-HSL Ser563 and P-HSL Ser660, particularly RW extract (Fig. 3).

JMP, SC and RCL decrease the size of adipocytes in cultured rat WAT. To confirm whether JMP, SC and RCL reduce the size of adipocytes, the cultured rat WAT was stained using HE staining. Histological analysis of the adipose tissue to confirmed the sizes of the adipocytes (Fig. 4). The size of adipocytes was markedly smaller in the ISO (750 nm) group, compared with that in the vehicle group. The adipocytes were also smaller in the 20 mg/ml JW extract, 200 mg/ml JE extract,

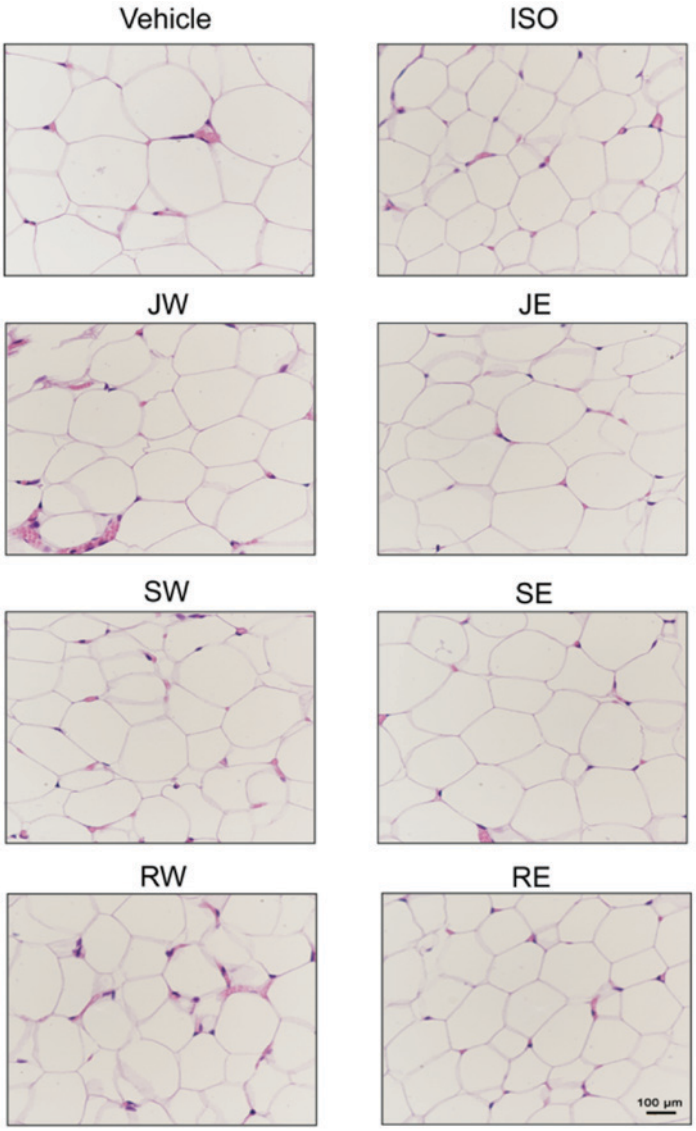

Figure 4. JMP, SC and RCL decrease the size of adipocytes in cultured rat WAT. The cultured rat WAT was stained using hematoxylin and eosin, and observed under a microscope (magnification, x400). The size of adipocytes is shown following treatment with JW, JE, SW, SE, RW and RE for 90 min. WAT, white adipose tissue; vehicle, normal saline; ISO, isoproterenol; JMP, jueming prescription; SC, semen cassiae; RCL, Rhizoma Curcumae Longae; JW, 20 mg/ml JMP water extract; JE, 200 mg/ml JMP ethanol extract; SW, 300 mg/ml SC water extract; SE, 300 mg/ml SC ethanol extract; RW, 400 mg/ml RCL water extract; RE, 400 mg/ml RCL ethanol extract.

300 mg/ml SW extract, 300 mg/ml SE extract, 400 mg/ml RW extract and 400 mg/ml RE extract groups, compared with size in the vehicle group.

Discussion

Obesity is characterized by excess TG accumulation in adipocytes of WAT (1). Lipolysis is the process of TG breakdown in WAT, resulting in the release of NEFA and glycerol (2-5,37). Experiments using cultured adipocytes, WAT explants and obese animals have demonstrated that increased phosphorylation levels of HSL at Ser563 and Ser660 promote lipolysis (20-22). Chinese herbal medicine is important in the treatment of obesity and enhancement of lipolysis (2,6,23-26). Our previous studies showed that JMP exhibited anti-obesity effects, and enhanced lipolysis in obese rats and patients (23,27,28). SC and RCL extracts also increased lipolysis to result in the release of NEFA and glycerol, and reduce the size of adipocytes (2,3,6,32). In the present study, it was found that JMP and its ingredients, SC and RCL, stimulated lipolysis and upregulated the phosphorylation levels of HSL at Ser563 and Ser660 in cultured rat WAT.

The induction of NEFA and glycerol release in intact WAT explants is used in the assessment of lipolysis (4,36,38-40). ISO, which is known to stimulate the release of NEFA and glycerol in rat WAT through the activation of β-adrenergic receptors, is used as a positive control (34-36). Therefore, the present study examined the release of NEFA and glycerol from ISO-, JMP-, SC- and RCL-treated rat WAT explants *in vitro*. ISO significantly increased the release of NEFA and glycerol. The JW, JE, SW, SE, RW and RE extracts also stimulated the release of NEFA and glycerol.

Previous studies have confirmed that the phosphorylation of HSL at Ser563 and Ser660 activates HSL, and increased HSL

translocates to lipid droplets and enhances lipolysis (19,20). In the present study, ISO, known to phosphorylate HSL at the Ser563 and Ser660 sites and increase lipolysis, was used as a positive control (21,22). It was found that the ISO, JW, JE, SW, SE, RW and RE extracts significantly upregulated the phosphorylation levels of HSL at Ser563 and Ser660 in the cultured rat WAT. The effects of the single herb SC and RCL extracts were superior to that of JMP. It was hypothesized that SC and RCL in JMP may induce a chemical reaction or the formation of certain compounds, leading JMP to have multiple effects and weakened lipolysis. This requires further investigation in the future. The RCL single herb had the most marked effect on increasing the levels of P-HSL Ser563 and P-HSL Ser660, particularly RW extract. This is consistent with the effects of water extracts of herbs used clinically for treating obese patients (27,41).

The present study also showed that ISO and the JW, JE, SW, SE, RW and RE extracts reduced the size of adipocytes in the cultured rat WAT. Histological analyses of the WAT confirmed that the sizes of the adipocytes were smaller on visualization under a microscope (magnification, x400) in the ISO-, JMP-, SC- and RCL-treated WAT, compared with the vehicle-treated group.

In conclusion, the results of the present study indicated that the effects of JMP and its ingredients, SC and RCL, on the stimulation of lipolysis were responsible for increases in the release of NEFA and glycerol, and a reduction in the size of adipocytes in an *ex vivo* organ culture method. This may be through upregulating the phosphorylation of HSL at Ser563 and Ser660 in rat WAT.

Acknowledgements

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