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ORIGINAL ARTICLE

Red mold rice prevents the development of obesity, dyslipidemia and hyperinsulinemia induced by high-fat diet

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Objective: To investigate the influences of red mold rice (RMR) on obesity and related metabolic abnormalities.
Methods and results: The 3T3-L1 cell line was used to examine the effects of RMR extracts on preadipocytes and on mature adipocytes. Both water and ethanol extracts of RMR had inhibitory effects on 3T3-L1 preadipocyte proliferation and differentiation. Water extracts of RMR enhanced the lipolysis activity in mature adipocytes, which negatively correlated with the triglyceride content within cells. RMR treatment did not affect heparin-releasable lipoprotein lipase activity in mature adipocytes. Furthermore, animal studies were carried out to explore the antiobesity effects of RMR. The control group of male Wistar rats were fed regular laboratory feed, whereas the other groups were fed the high-fat (HF) diet supplemented with lovastatin, rice or RMR (0.4 and 2%, $w\,w^{-1}$). The relative caloric intakes of the control and HF groups were 3.34 and 4.85 $kcal\,g^{-1}$, respectively. After 6 weeks, rats treated with RMR at the 0.4 and 2% doses had lower weight gain and less fat pads mass accompanied with smaller fat cells than did the HF-diet rats. These effects probably resulted from an increase in the lipolysis activity of adipose tissue and a reduction in food/energy consumption. On the other hand, the RMR supplement significantly reduced serum total cholesterol, serum low-density lipoprotein (LDL) cholesterol, the ratio of LDL to high-density lipoprotein (HDL) cholesterol and serum insulin in the HF group. Moreover, the 2% RMR treatment significantly increased serum HDL cholesterol.
Conclusion: This study reveals for the first time that RMR can prevent body fat accumulation and improve dyslipidemia. The antiobesity effects of RMR mainly derive from the lipolytic activity and mild antiappetite potency of RMR. In addition, extracts of RMR suppressed the proliferation and differentiation in 3T3-L1 preadipocytes, which might have contributed to the inhibition of new adipocyte formation or hyperplasia in adipose tissue.
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Keywords: 3T3-L1 preadipocytes; red mold rice; high-fat diet; dyslipidemia

Introduction

Obesity is defined as an excess of white adipose tissue, which is associated with a higher risk of developing diabetes and cardiovascular disease. At the cellular level, enlargement of the adipose tissue mass has been characterized by an increase in the size (hypertrophy) or number (hyperplasia) of adipocytes. The triglyceride (TG) content in adipocytes reflects the balance between lipogenesis and lipolysis, which is largely related to cell volume. When adipocytes reach a critical size threshold, preadipocytes in close proximity to the adipocytes will respond to positive energy balance by proliferating and then differentiating into adipocytes to store the excess energy.[1] Early in life, adipose tissue expansion occurs primarily through hyperplasia.[2] However, humans and rodents have the capacity to form new fat cells from preadipocytes throughout life.[3,4] Several mechanisms reduce the risk of obesity, including reduced energy/food intake, decreased intestine adsorption, suppressed lipogenesis, enhanced lipolysis and fat oxidation, increased energy expenditure and inhibited preadipocyte proliferation and differentiation.

Red mold rice (RMR) is produced through the fermentation of ordinary rice with *Monascus* species. In East Asian countries, RMR traditionally functioned as a flavoring, coloring and preservative in food and as a medicinal herb for improving digestion and revitalizing the blood.[5] Many studies have shown that some secondary metabolites

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of *Monascus* species improved hyperlipidemia,[6,7] hypertension,[8] hyperglycemia,[9,10] tumor promotion,[11] Alzheimer's disease development,[12,13] oxidative stress[14,15] and fatigue during exercise.[16]

Monacolin K (lovastatin), a metabolite produced by *Monascus*, has been characterized as a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (statins) with cholesterol-lowering activity. Numerous studies have reported that statins have the ability to regulate adipocyte differentiation.[17–19] Recently, Jeon *et al*. demonstrated that water extracts of RMR suppress adipogenesis in 3T3-L1 preadipocytes through the downregulated expression of adipogenic transcription factors and other specific genes.[20] On the basis of these findings, we suggest that RMR could decrease the adipose tissue mass and might alleviate the progression of obesity.

In this study, we used the 3T3-L1 cell line as an *in vitro* model to determine the effects of RMR on proliferation and differentiation in preadipocyte, lipolysis and the activity of heparin-releasable lipoprotein lipase (HR-LPL)—a key enzyme responsible for lipogenesis—in mature adipocyte. Furthermore, we used an animal model to investigate the antiobesity effects of RMR *in vivo*.

Materials and methods

Reagents and cells

3T3-L1 preadipocytes were kindly provided by Dr F Sheu (National Taiwan University, Taipei, Taiwan, ROC). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Dexamethasone, isobutylmethylxanthine, insulin, lovastatin, oil-red *O*, heparin, *p*-nitrophenyl butyrate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Trypan blue stain was purchased from Gibco BRL Life Technologies Inc. (Gaithersburg, MD, USA).

Preparation of red mold rice extracts

RMR was made by fermenting embryonic rice with *Monascus purpureus* NTU 568 as described earlier.[21] The dried sample was extracted with water at 37 °C for 24 h and then filtered. The filtrate was freeze-dried for preparation of the water extract (RMR-W), whereas the pellet was further extracted with ethanol at 37 °C for 24 h. After filtration, the ethanol elute was collected and then evaporated for preparation of ethanol extracts (RMR-E).

Cell culture

3T3-L1 preadipocytes were cultured in basal medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum) at 37 °C in 5% CO_2. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone and 10 μg ml^{-1} insulin (MDI) added to basal medium. On day 2, the MDI medium was replaced with basal medium containing insulin only. On day 4 and thereafter, the cells were cultured in basal medium, which was freshly changed every 2 days until the cells were analyzed. RMR-W and RMR-E were dissolved in phosphate-buffered saline and ethanol, respectively, and then added to culture medium at final concentrations of 50, 100 and 200 μg ml^{-1}. The vehicle control was 0.3% ethanol in culture medium, which was equal to the ethanol concentration in the highest-dose RMR-E treatment (200 μg ml^{-1}).

Proliferation assay

Preconfluent 3T3-L1 preadipocytes were seeded in 24-well dishes at a density of 7.5×10^3 cell per 0.5 ml per well. After the cells adhered to the dishes, extracts of RMR were added to the culture medium at the indicated doses for 24 and 48 h. Viable cells at each dose and time point were evaluated by MTT colorimetric assay.[22]

Cytotoxicity assay

Cytotoxicity was evaluated *in vitro* by determining cell viability using the trypan blue exclusion method.[23] This method is based on the principle that dead cells are not able to exclude trypan blue and thus appear blue. 3T3-L1 preadipocytes were collected 24 and 48 h after being treated with extracts of RMR and were then incubated with 0.4% trypan blue. Cells were observed under a microscope and counted as stained or nonstained cells separately with a hemocytometer. The viable cell ratios were calculated according to the following formula: viability (%) = (nonstained cells number/total cells number) × 100%.

Differentiation assay

The extracts of RMR were added to the culture medium throughout the differentiation process. On day 8 after the initiation of differentiation, 3T3-L1 cells were scraped in 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1% Triton X-100 and then assayed to determine the TG content using a commercial kit (TR213; Randox Inc., Antrim, UK). The results were normalized by using cellular protein (BCA protein kit; Pierce Biotechnology Inc., Rockford, IL, USA). For oil-red O staining, differentiated 3T3-L1 cells on day 8 were fixed with 10% formaldehyde and then stained with oil-red O. Pictures were taken using a microscope (ECLIPSE TS100; Nikon Co., Tokyo, Japan).

Lipolysis assay

The fully differentiated 3T3-L1 adipocytes (days 8–12 after differentiation induction) were treated with extracts of RMR in Krebs Ringer bicarbonate (KRB) buffer (20 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 2% BSA; pH 7.4) for 24 h. Lipolysis activity was determined by measuring the amount

of glycerol released into the incubation medium (GY105; Randox Inc.). Furthermore, the cells were scraped off for measurement of the TG content. In the animal study, 0.1 g adipose explants of perirenal and epididymal fat pads from experimental rats were incubated in 1 ml of KRB buffer at 37 °C for 1 h. Glycerol was determined enzymatically from the supernatant by using a Randox kit.[24]

HR-LPL activity assay

After incubation of the 3T3-L1 mature adipocytes with the experimental medium for 24 h, the medium was discarded. The cells were rinsed with KRB buffer and then cultured in heparin-KRB (10 U ml^{-1} heparin) at 37 °C for 1 h. The conditioned heparin-KRB was collected from each well for the assay of HR-LPL activity. In the animal study, a sample of perirenal and epididymal adipose tissue weighing 0.1 g was placed in 1 ml of KRB buffer supplemented with 10 U ml^{-1} heparin at 37 °C for 1 h.[25]

LPL activity was measured on the basis of its esterase property using *p*-nitrophenyl butyrate as a substrate.[26,27] The TG hydrolase activity of LPL with synthetic TG substrates is inhibited by molar sodium chloride,[28] and this property has been used to distinguish LPL activity from the activities of other lipases in plasma.[29,30] Thus, HR-LPL activity was calculated from the productivity of *p*-nitrophenol using the following equation:

$$C(\mu M) = \frac{A_{400(0.15\,M\,NaCl)} - A_{400(1\,M\,NaCl)}}{0.012}$$

where $A_{400(0.15\,M_{NaCl})}$ and $A_{400(1\,M_{NaCl})}$ are the absorbances of released *p*-nitrophenol at 400 nm in 0.15 M and in 1 M NaCl assay buffer, respectively, and 0.012 is the micromolar extinction coefficient of *p*-nitrophenol.

Animal experiments

Male Wistar rats at 6–8 weeks of age were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). The animals were housed individually and allowed free access to a standard laboratory chow (Ralston Purina, St Louis, MO, USA) and water. Three weeks later, the rats were randomly assigned to one of the following diets for 6 weeks: standard chow (control group; 4.5% fat, 3.34 kcal g^{-1}), high-fat (HF) diet consisting of 26.7% butter powder (Gene Asia Biotech Co., Ltd., Nang-Tou, Taiwan) in standard chow (HF group; 30% fat, 4.85 kcal g^{-1}), HF + 1.43 mg kg day^{-1} lovastatin (L group), HF + 0.4% rice (R group), HF + 0.4% red mold rice (RMR-L group) or HF + 2% red mold rice (RMR-H group). The doses of RMR used in this study were calculated according to Boyd's formula for body surface area,[31] such that a daily dose of 0.4% RMR in rats is equivalent to a daily supplement of 2 g of RMR in adult humans (weight: 65 kg; height: 170 cm)—the usual recommended daily dietary dose of RMR. The dose of lovastatin in the L group was equal to the monacolin K content of RMR in the RMR-L group. Food consumption and body weight were recorded weekly. Feces were collected from rats on three consecutive days and oven dried (65 °C) to a constant weight for the determination of fat content. At the end of the study, the rats were deprived of food for 16 h before being killed by CO_2 asphyxiation. Blood samples were collected from the posterior vena cava and centrifuged at 700 × *g* for 10 min; the serum was stored at −20 °C until analyzed. Perirenal and epididymal fat pads were removed and weighed. Portions of the adipose tissue were immersed in 10% formaldehyde for histological inspection; other portions were frozen immediately in liquid nitrogen and stored at −80 °C for analysis of lipolysis and HR-LPL activity. Liver was excised and stored at −20 °C for the measurement of lipids.

Analyses

Whole blood glucose was assayed immediately using a EUSURE blood glucose monitoring system (Eumed Biotechnology Co. Ltd., Hsinchu, Taiwan). The liver samples were homogenized and extracted with a 2:1 (v v^{-1}) mixture of chloroform: methanol for the measurement of TG and total cholesterol (TC) concentrations.[32] The contents of TG, TC and high-density lipoprotein (HDL) cholesterol (HDL-C) in serum or liver were measured using the commercial kits (Randox Inc.). Low-density lipoprotein cholesterol (LDL-C) was estimated according to the following formula: LDL-C = TC−HDL-C−(TG/5).[33] An enzyme-linked immunosorbent assay kit was used to measure serum insulin (rat insulin enzyme-linked immunosorbent assay; Mercodia, Uppsala, Sweden). The activity of glutamic oxaloacetic acid transaminase and glutamic pyruvic acid transaminase as well as the concentrations of creatinine, uric acid, Na^+ and K^+ in serum were analyzed with an Autobiochemistry Assay System (Beckman-700; Beckman, Fullerton, CA, USA). Fecal lipids were extracted by using the method of Folch *et al*.[32] The total fat content in feces was determined gravimetrically. On the basis of the 3-d intake and excretion data, apparent fat absorption was calculated using the following formula: Apparent fat absorption (%) = ((fat intake−fecal fat)/fat intake) × 100%.[34]

Adipose tissue histology

The adipose tissue samples were fixed in formaldehyde, embedded in paraffin, cut into 5-μm sections and stained with hematoxylin and eosin. Cross-sectional areas of the adipocytes were calculated from the histogram according to Chen and Farese.[35] For the estimation of fat pads cell number, the lipid content of 0.3 g of fat tissue was extracted by using the method of Folch *et al*.[32] The total cell number in the fat pads was calculated by dividing the lipid content of the fat pad by the mean weight of cell lipids. The lipid weight of the average fat cell was calculated from the mean cell volume, assuming a lipid density of 0.915 (density of triolein).[36]

Statistical analysis
The results are expressed as means ± s.d. Analysis of variance was used for the statistical analysis followed by Duncan's multiple-range test. Statistical significance was accepted at $P<0.05$. A correlation analysis was performed using Pearson's test.

Results

Experiment 1: cellular studies
Proliferation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured in the presence and absence of RMR-W or RMR-E at the indicated doses. Cell proliferation was determined by MTT at 24 and 48 h after incubation. As shown in Figure 1, both RMR-W and RMR-E inhibited cell growth in 3T3-L1 preadipocytes in a dose-dependent manner. At 48 h, 200 μg ml^{-1} RMR-W and RMR-E inhibited proliferation by nearly 22.2 and 31.8%, respectively.

To determine whether monacolin K reduced proliferation in preadipocytes, we treated the 3T3-L1 preadipocytes with various doses of lovastatin for 24 h. Lovastatin had an inhibitory effect on 3T3-L1 preadipocyte proliferation (Figure 1c). Monacolin K is virtually insoluble in water and was mainly present in RMR-E (data not shown), which may be an antiproliferative component of RMR-E.

Cytotoxicity assay. The results demonstrated that treatment with 50–200 μg/ml of RMR-W or RMR-E showed no cytotoxicity on 3T3-L1 preadipocytes (Table 1). Therefore, the suppressive property of the RMR extracts in 3T3-L1 preadipocyte proliferation may have been the result of growth retardation instead of cytotoxicity.

Differentiation in 3T3-L1 preadipocytes. Postconfluent 3T3-L1 preadipocytes were exposed to RMR-W or RMR-E during the induction of differentiation. On day 8 after the onset of differentiation, cells were harvested for measurement of the TG content—a marker of adipogenesis. As shown in Figure 2a, RMR-W significantly decreased intracellular TG accumulation. However, 50–200 μg ml^{-1} RMR-W seemed to have similar inhibitory effects on adipogenesis in 3T3-L1

Table 1 Cytotoxic effects of extracts of *Monascus* fermented products

Concentration (μg ml^{-1})	*Cell viability (%)*	
	RMR-W	*RMR-E*
Control	95.70 ± 1.29	98.72 ± 1.95
50	96.79 ± 3.31	97.73 ± 0.63
100	94.62 ± 0.37	98.76 ± 0.44
200	94.20 ± 2.23	94.84 ± 0.49

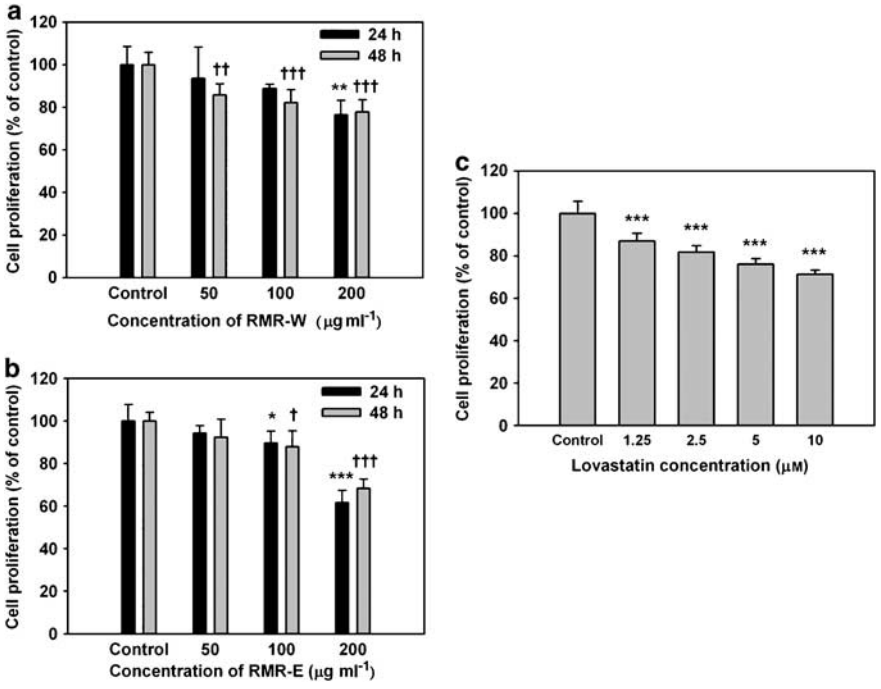

Figure 1 Effects of (**a**) red mold rice water extracts (RMR-W), (**b**) red mold rice ethanol extracts (RMR-E) and (**c**) lovastatin on 3T3-L1 preadipocyte proliferation. Preadipocytes were incubated with RMR extracts or lovastatin at the indicated concentrations for 24 and 48 h. The degree of proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and expressed as a percentage of the (vehicle) control. The results are expressed as the mean ± s.d. ($n=4$). Duncan's test was used for the statistical analysis. Significantly different from (vehicle) control group at 24 h: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Significantly different from (vehicle) control group at 48 h: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$.

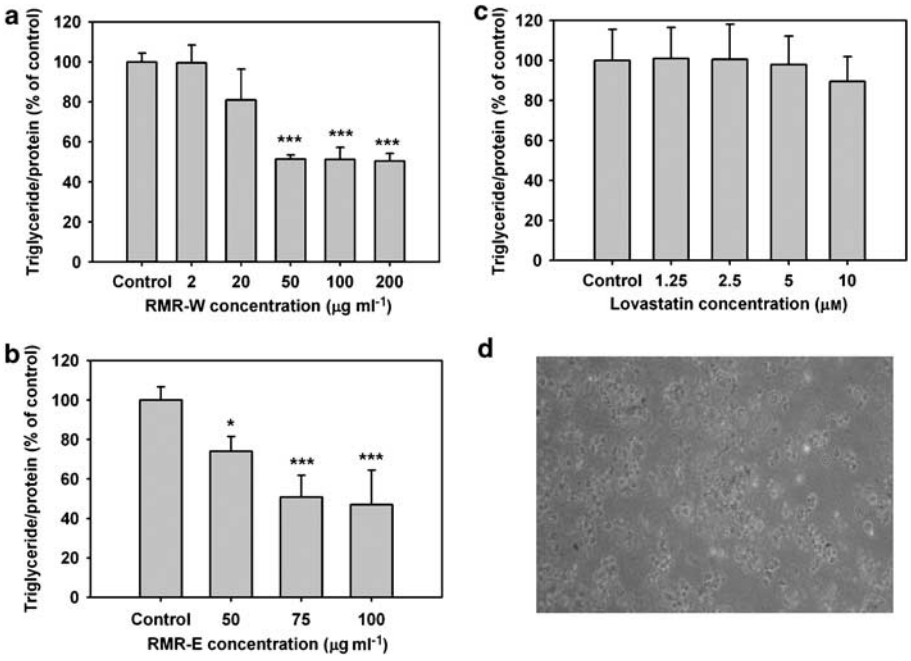

Figure 2 Effects of red mold rice (RMR) extracts and lovastatin on 3T3-L1 preadipocyte differentiation. Preadipocytes were differentiated according to the method described in 'Materials and methods.' During differentiation the cells were treated with (**a**) RMR water extracts (RMR-W), (**b**) RMR ethanol extracts (RMR-E) or (**c**) lovastatin at the indicated concentrations. On day 8, the cells were harvested to determine triglyceride accumulation—a marker of adipogenesis. The results are expressed as a percentage of the (vehicle) control as the mean ± s.d. ($n=3$). Duncan's test was used for the statistical analysis. Significantly different from (vehicle) control group: $^{*}P<0.05$, $^{***}P<0.001$. (**d**) 3T3-L1 preadipocytes detached from the culture dishes after incubation with 200 μg ml^{-1} RMR-E.

preadipocytes, because the TG content within cells decreased approximately by 50% compared with controls.

RMR-E inhibited the adipogenesis of 3T3-L1 preadipocytes in a dose-dependent manner. Cells treated with 100 μg/ml of RMR-E accumulated nearly 53% of the intracellular TG contained in controls (Figure 2b). It was noted that the cells became round and detached from the culture dishes in the presence of higher concentrations of RMR-E (Figure 2d). Similar morphological changes were observed in a previous study in which 3T3-L1 cells were treated with 10 μM simvastatin during the early phase (days 0–2) of adipogenesis.[19] Simvastatin caused this change as early as 6 h after the induction of differentiation and it had no such effect on undifferentiated preadipocytes or preadipocytes during the late adipogenesis phase (days 2–4).[19] It appears that the detachment of 3T3-L1 cells at higher doses of RMR-E was partially because of the increased monacolin K component. To verify whether monacolin K inhibited adipogenesis, we cultured cells in the presence or absence of lovastatin at the indicated concentrations and examined the intracellular TG content. However, lovastatin at 10 μM only decreased TG accumulation by 11% (Figure 2c). The results indicated that the antiadipogenic activity of RMR-E might have been due to other components.

In addition to the TG content, oil-red O staining was used as an indicator of adipogenesis. The microscopic images demonstrated that both RMR-W and RMR-E attenuated lipid accumulation in 3T3-L1 preadipocytes, which had antiadipogenic activity against 3T3-L1 cells (Figure 3).

Lipolysis activity in mature 3T3-L1 adipocytes. Extracts of RMR were added to differentiated 3T3-L1 cells for 24 h, and glycerol released into the culture medium was measured as the index of lipolysis activity in cells. RMR-W exerted moderate lipolytic activity in a dose-dependent manner, whereas RMR-E or lovastatin had no significant effects on lipolysis (Figure 4). RMR-W at doses of 50, 100 and 200 μg ml^{-1} increased glycerol release by 33, 41 and 49%, respectively, and the TG content in treated cells was lower than that in the control cells. There was a negative correlation between lipolysis activity and cellular TG ($r=-0.715$, $P<0.01$) (Figure 4d).

HR-LPL activity in mature 3T3-L1 adipocytes. Neither RMR extracts nor lovastatin affected the HR-LPL activity in 3T3-L1 adipocytes (data not shown).

Experiment 2: animal studies

Body weight gain, food/energy intake, feed efficiency and apparent fat absorption in male Wistar rats. After 6 weeks of feeding, the rats fed the HF diet gained more weight than did those fed the standard laboratory chow ($P<0.001$; Table 2). RMR at 0.4 and 2% (w w^{-1}) in the HF diet significantly suppressed

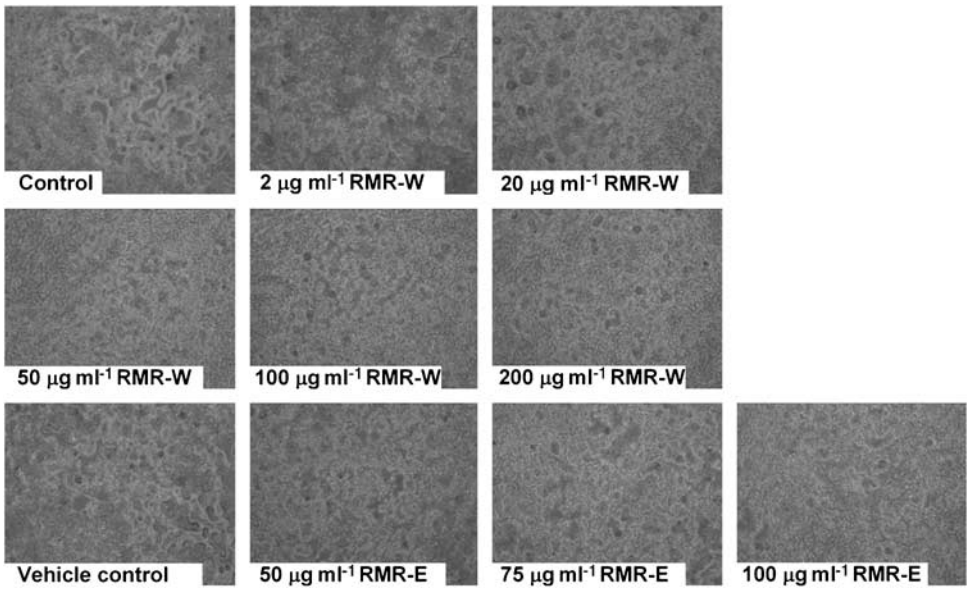

Figure 3 Effects of red mold rice water extracts (RMR-W) and red mold rice ethanol extracts (RMR-E) on 3T3-L1 preadipocyte differentiation. Preadipocytes were differentiated according to the method described in 'Materials and methods.' During differentiation the cells were treated with RMR-W or RMR-E at the indicated concentrations. On day 8, the cells were fixed and stained for triglyceride measurement with oil-red O.

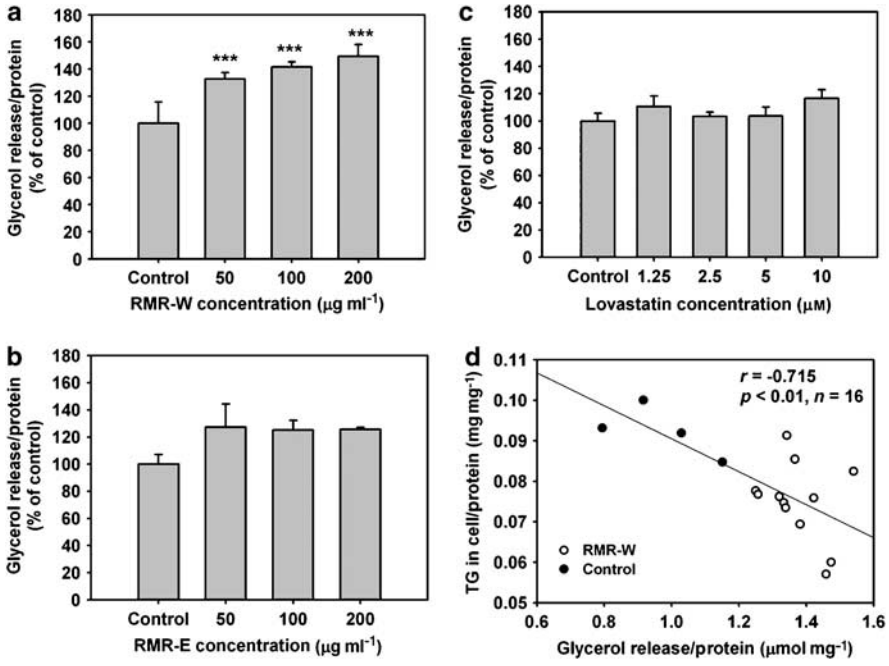

Figure 4 Effects of red mold rice (RMR) extracts and lovastatin on lipolysis activity in 3T3-L1 adipocytes. Preadipocytes were differentiated according to the method described in 'Materials and methods.' Fully differentiated cells were treated for 24 h with (**a**) RMR water extracts (RMR-W), (**b**) RMR ethanol extracts (RMR-E) or (**c**) lovastatin at the indicated concentrations. The conditioned medium was then collected and assayed for glycerol content. The results are expressed as a percentage of the (vehicle) control as the mean ± s.d. ($n=4$). Duncan's test was used for the statistical analysis. Significantly different from (vehicle) control group: ***$P<0.001$. (**d**) Correlation between glycerol release and intracellular triglyceride (TG) content of the cells treated (○) or untreated (●) with RMR-W. The degree of the linear relationship between two variables was measured by Pearson's correlation coefficient.

the increase in weight gain by 21.5% ($P<0.05$) and 30.5% ($P<0.01$), respectively. Consumption of the HF diet resulted in a decrease in food consumption; however, energy intake in the HF group was 22.4% ($P<0.001$) higher than that in the control group. In accordance with a previous study[37] in which lovastatin reduced the food/energy consumption by

Table 2 Effects of RMR supplement on the body weight gain, food/energy intake, feed efficiency and fat absorption in male Wistar rats fed high fat diet

	Weight gain (g)	*Food intake (g d^{-1})*	*Energy intake (kcal d^{-1})*	*Feed efficiency (%)*	*Fat absorption (%)*
C	115.3 ± 24.0***	26.85 ± 1.23***	89.7 ± 4.1***	10.20 ± 2.0***	81.51 ± 5.51***
HF	171.0 ± 25.4	22.64 ± 1.89	109.8 ± 9.2	17.94 ± 1.64	95.84 ± 0.72
L	141.1 ± 35.3	20.21 ± 2.15*	98.0 ± 10.4*	16.48 ± 3.02	96.08 ± 1.12
R	156.5 ± 41.3	22.06 ± 1.32	107.0 ± 6.4	16.73 ± 3.35	95.51 ± 1.03
RMR-L	134.3 ± 20.7*	21.14 ± 1.56	102.5 ± 7.6	15.09 ± 1.75*	95.17 ± 2.14
RMR-H	118.9 ± 27.0**	19.71 ± 1.29**	95.6 ± 6.2**	14.30 ± 2.75**	96.10 ± 0.69

Data are expressed as means ± s.d. ($n = 8$). Duncan's test: *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly from the value of the HF group.

Table 3 Effects of RMR supplement on the weight and cellularity of perirenal and epididymal adipose tissues in male Wistar rats fed high fat diet

	Perirenal fat pads			*Epididymal fat pads*		
	Total weight (g)	*Cell cross-sectional area (μm^2)*	*Cell number $\times 10^6$/ total tissue*	*Total weight (g)*	*Cell cross-sectional area (μm^2)*	*Cell number $\times 10^6$/ total tissue*
C	7.0 ± 2.0***	5108.2 ± 1153.8**	16.8 ± 7.9	7.7 ± 1.4***	3812.1 ± 567.5***	29.0 ± 5.0
HF	13.2 ± 3.9	7527.5 ± 1708.3	21.7 ± 8.5	11.9 ± 2.9	5209.6 ± 747.7	31.1 ± 6.7
L	10.4 ± 1.1	6636.4 ± 559.0	18.3 ± 5.6	9.5 ± 1.4*	4600.6 ± 811.8*	28.8 ± 9.1
R	11.6 ± 1.5	7319.2 ± 1529.8	21.6 ± 17.8	11.4 ± 1.7	5163.9 ± 1069.9	31.6 ± 12.6
RMR-L	9.7 ± 2.0*	5842.2 ± 1369.1*	21.3 ± 9.1	9.5 ± 1.4*	4158.7 ± 537.3**	33.8 ± 10.7
RMR-H	8.1 ± 2.0***	5361.8 ± 1289.6**	20.3 ± 7.9	8.8 ± 2.2*	4021.5 ± 206.8***	30.7 ± 11.2

Data are expressed as means ± s.d. ($n = 8$). Duncan's test: *$P<0.05$, **$P<0.01$, ***$P<0.001$, significantly from the value of the HF group.

10.7% ($P<0.05$), lovastatin reduced the food/energy consumption by 12.9% ($P<0.01$) in this study, but only in the high-dose RMR group (2%, w w^{-1}).

Feed efficiency was defined as grams of weight gained per gram of diet. The HF diet, which provided more fat and energy than the other diets, generated greater ($P<0.001$) feed efficiency than the standard feed diet. The addition of RMR to the HF diet at levels of 0.4 and 2% significantly decreased the feed efficiency ($P<0.05$ and $P<0.01$, respectively; Table 2). The results suggest that RMR might inhibit weight gain in rats through effects other than reductions in food intake.

The lipid absorption capability of rats that were fed standard feed was significantly ($P<0.001$) lower than that in rats that were fed the HF diet, possibly because of the inducing effects on both the intestinal mitotic index and on the expression of genes involved in fatty acid uptake, trafficking and lipoprotein synthesis with the HF diet.[38] Neither RMR nor lovastatin affected apparent fat absorption in the rats that were fed the HF diet (Table 2).

There was no significant difference in body weight gain, food/energy intake, feed efficiency or fat absorption between the R group and the HF group (Table 2), which suggests that the addition of 0.4% rice to the HF diet did not have an obvious influence on the nutrient status of rats.

Weight and cellularity of adipose tissues in male Wistar rats. The effects of diet on adipose tissue characteristics are shown in Table 3. Rats fed the HF diet showed a significant increase in weight ($P<0.001$) and cell size of perirenal ($P<0.01$) and epididymal ($P<0.001$) adipose tissues. No significant differences in cell numbers in either type of adipose tissue were noted between the diet groups. It appears that the increased size rather than number of adipocytes contributed to the increase in fat mass in the HF group. Treatment with 0.4 and 2% RMR caused a reduction in the weight of perirenal (by 26.5 and 38.6%, respectively) and epididymal (by 20.2 and 26.1%, respectively) adipose tissues. Similarly, a decrease in the cross-sectional area of fat cells was observed. Finally, the weight and cell size of epididymal adipose tissue in the L group were significantly ($P<0.05$) smaller than those in the HF group. Thus, monacolin K might partially account for the antiobesity effects of RMR.

Lipolysis and HR-LPL activity of adipose tissue in male Wistar rats. As shown in Figures 5a and b, the administration of 0.4 and 2% RMR to the HF group significantly increased the lipolysis activity in perirenal (12.3 and 17.3%, respectively) and epididymal (29.0 and 30.0%, respectively) adipose tissues. On the other hand, rats fed standard feed exhibited lower lipolysis activity in perirenal adipose tissue than did those that were fed the HF diet ($P<0.001$).

The HF diet significantly increased the HR-LPL activity of perirenal and epididymal adipose tissues in rats ($P<0.001$ and $P<0.05$, respectively). It has been shown that LPL activity in adipose tissue is upregulated by a chronic HF diet, whereas skeletal muscle LPL activity is downregulated.[39] This biphasic pattern results in dietary fat being diverted

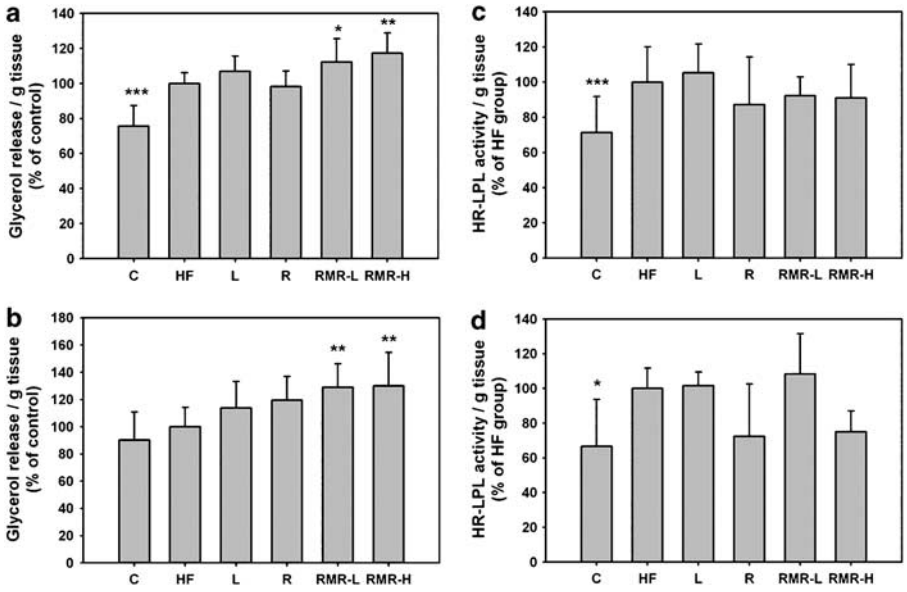

Figure 5 Effects of red mold rice (RMR) supplementation on lipolysis and heparin-releasable lipoprotein lipase (HR-LPL) activity in perirenal (**a** and **c**) and epididymal (**b** and **d**) adipose tissue in male Wistar rats fed a high-fat (HF) diet. Rats were fed a standard chow control diet (**c**) or the chow diet supplemented with butter (HF), lovastatin (L), 0.4% rice (R), 0.4% RMR (RMR-L) or 2% RMR (RMR-H). Lipolysis was estimated as the amount of glycerol released in the medium from 0.1 g of tissue. HR-LPL activity was evaluated as the amount of LPL released in heparin buffer from 0.1 g of tissue. The results are expressed as a percentage of the HF group as the mean ± s.d. ($n=8$). Duncan's test was used for the statistical analysis. Significantly different from the HF group: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 4 Effects of RMR supplement on the lipidic and glycemic parameters in male Wistar rats fed high fat diet

	Serum TC (mg per 100 ml)	*Serum TG (mg per 100 ml)*	*Serum LDL-C (mg per 100 ml)*	*Serum HDL-C (mg per 100 ml)*	*Serum LDL-C/ HDL-C*	*Hepatic TC (mg per 100 ml)*	*Hepatic TG (mg per 100 ml)*	*Serum insulin (pmole l^{-1})*
C	52.6 ± 8.5*	76.6 ± 18.8	8.1 ± 3.9*	29.2 ± 7.7*	0.3 ± 0.1**	63.6 ± 7.4	259.7 ± 37.3	86.7 ± 34.1**
HF	65.2 ± 5.7	94.6 ± 14.1	23.9 ± 6.7	22.4 ± 3.6	1.1 ± 0.4	64.3 ± 13.9	304.2 ± 74.2	131.6 ± 37.1
L	49.0 ± 4.2*	87.6 ± 15.8	9.0 ± 2.6*	22.4 ± 3.8	0.4 ± 0.1*	58.1 ± 5.0	350.2 ± 44.5	106.0 ± 28.3
R	65.2 ± 8.8	96.7 ± 26.9	23.5 ± 5.0	22.4 ± 4.1	1.1 ± 0.3	63.8 ± 13.7	366.3 ± 96.3	135.2 ± 15.2
RMR-L	51.5 ± 5.7*	75.4 ± 11.9	9.1 ± 2.2*	27.8 ± 4.9	0.3 ± 0.2**	59.4 ± 10.9	341.1 ± 41.5	92.2 ± 27.0*
RMR-H	52.6 ± 6.5*	82.6 ± 10.6	6.0 ± 1.9**	30.0 ± 4.8*	0.2 ± 0.1**	55.0 ± 8.3	317.2 ± 49.6	89.7 ± 25.5**

Data are expressed as means ± s.d. ($n=8$). Duncan's test: *$P<0.05$, **$P<0.01$, significantly from the value of the HF group.

into adipose tissue for storage. Treatment with RMR had no effect on HR-LPL activity in either type of adipose tissue (Figures 5c and d).

Lipid and glucose variables in Wistar rats. Serum TC, LDL-C and the ratio of LDL-C to HDL-C were significantly greater and HDL-C was significantly lower in the HF group than in the control group (Table 4). Addition of RMR or lovastatin to HF diet significantly suppressed the increase in serum TC, LDL-C and LDL-C/HDL-C ratio. Moreover, the 2% RMR treatment generated a significant rise in serum HDL-C. Serum TG and hepatic TC and TG did not change significantly in any of the diet groups.

No significant difference in blood glucose was observed between the groups (data not shown). However, serum insulin levels were significantly greater in the HF group than in the control group. Several researches have shown that diets high in fat and/or in sucrose tend to induce insulin resistance or hyperinsulinemia in animals,[40,41] which is the underlying defect that ultimately leads to the development of the other metabolic syndromes, including hypertriglyceridemia, hypertension and obesity.[42,43] Hyperinsulinemia induced by the HF diet improved with the RMR treatments (Table 3).

On the other hand, serum glutamic oxaloacetic acid transaminase, glutamic pyruvic acid transaminase, creatinine, uric acid, Na^+ and K^+ had no significant effects on the HF diet or RMR treatment (Table 5).

Discussion

In cellular studies, we reported that either water or ethanol extracts of RMR had inhibitory effects on proliferation and adipogenesis in 3T3-L1 preadipocytes. In addition, water

Table 5 Serum parameters of male Wistar rats fed the high-fat diet after 6 weeks of treatment with *Monascus* fermented products

	GOT ($U l^{-1}$)	*GPT ($U l^{-1}$)*	*Creatinine (mg per 100 ml)*	*Uric acid (mg per 100 ml)*	*Na^+ ($mEq l^{-1}$)*	*K^+ ($mEq l^{-1}$)*
C	79.79 ± 10.50	49.63 ± 9.81	0.35 ± 0.03	3.35 ± 0.60	148.9 ± 1.63	5.39 ± 0.57
HF	75.21 ± 9.60	45.50 ± 9.91	0.36 ± 0.04	3.36 ± 1.08	151.3 ± 7.88	5.44 ± 0.42
L	73.63 ± 8.13	47.83 ± 5.80	0.32 ± 0.04	3.27 ± 0.56	147.1 ± 3.03	5.55 ± 0.61
R	73.08 ± 6.77	47.00 ± 6.22	0.37 ± 0.04	3.99 ± 1.29	147.4 ± 1.85	6.11 ± 1.26
RMR-L	71.88 ± 3.88	45.13 ± 5.04	0.33 ± 0.04	3.89 ± 1.64	148.7 ± 1.21	6.17 ± 1.25
RMR-H	73.29 ± 10.15	49.75 ± 13.18	0.34 ± 0.04	4.12 ± 1.72	148.2 ± 1.15	5.86 ± 0.83

extracts of RMR promoted lipolysis activity and attenuated the accumulation of TG in 3T3-L1 mature adipocytes; however, treatment with RMR extracts had no significant effect on HR-LPL activity. To better assess the role of RMR in fat deposition and body weight management, the animal studies were carried out. RMR-supplemented rats had lower body weight gain and lower fat pad mass accompanied by smaller fat cells than did the HF-diet rats, and apparent fat absorption and HR-LPL activity in the adipose tissues did not change significantly. The 0.4 and 2% RMR treatments significantly enhanced the lipolysis activity of adipose tissues, which was consistent with the findings of the *in vitro* experiment. It is possible that the antiobese effects of RMR partially resulted from lipolytic activity. Moreover, rats fed 2% RMR consumed less food/energy, which may have further influenced the antiobese effects of RMR. Although hyperplasia of adipocytes was not observed in our HF-diet rat model, the cellular studies revealed that RMR extracts exerted antiproliferative and antiadipogenic effects on preadipocytes, which contributed to the suppression of new adipocyte formation and related adipose tissue enlargement.

RMR, a common fermented product of *Monascus*, has been used as a traditional cuisine and medicinal agent in Eastern Asia for several centuries. Currently, RMR is recognized as a functional food because of the proven benefits of some of its secondary metabolites, such as monacolins (cholesterol-lowering agents),[44] γ-aminobutyric acid (hypotensive agent),[45] azaphilone pigments (anti-inflammatory agents)[46] and dimerumic acid, tannin, phenol and unsaturated fatty acids (antioxidants).[14,15] Monacolin K, also known as lovastatin, is usually the most abundant monacolin in RMR. Besides its ability to lower the cholesterol level, the competitive inhibitory effect of statins on HMG-CoA reductase, the major rate-limiting enzyme that controls the conversion of HMG-CoA to mevalonic acid, results in pleiotropic effects on many essential cellular functions, including cell proliferation, differentiation, survival and participation in the regulation of cell shape and motility.[47]

In this study, lovastatin treatment caused a moderate decrease in 3T3-L1 preadipocyte proliferation, which might account, at least in part, for the antiproliferative activity of RMR-E. However, the water extracts of RMR also showed an inhibitory effect on 3T3-L1 preadipocyte proliferation, suggesting that components of RMR other than monacolin K may also have antiproliferative activity against preadipocytes.

Unexpectedly, the results of our studies were not consistent with those of previous researches; lovastatin treatment did not significantly affect adipogenesis in 3T3-L1 preadipocytes up to 10 μM. Jeon *et al.*[20] added 50 $\mu g\,ml^{-1}$ lovastatin to 3T3-L1 preadipocytes to clarify the antiadipogenic ingredients of RMR extracts, however; lovastatin showed a marginal influence on adipogenesis at this dose. More studies are required to examine the effects of lovastatin on 3T3-L1 differentiation. These data suggest that the inhibitory effect of RMR-E on adipogenesis may be due to the bioactive ingredients of compounds other than monacolin K. Moreover, when 3T3-L1 preadipocytes were incubated with RMR-E at doses higher than 100 $\mu g\,ml^{-1}$, the cells became round and detached from the culture dishes. According to a previous study,[19] this morphological change might partially be the result of a higher monacolin K content, although the phenomenon was not observed in undifferentiated preadipocytes or in fully differentiated adipocytes subjected to the same RMR-E treatment. At the beginning of differentiation, 3T3-L1 preadipocytes undergo one or two additional rounds of cell division known as clonal expansion. It is likely that the high dose of statins inhibited postconfluent mitosis and hindered the growth of preadipocytes in the early adipogenesis phase. This presumption should be tested in future experiments.

Differentiated adipocytes incorporate glucose and free fatty acids to synthesize and accumulate TG as energy. The TG content within cells reflects the balance between lipogenesis and lipolysis, which is also responsible for the determination of cell size. To verify whether RMR has the potential to prevent the hypertrophy of fat cells, we cultured differentiated 3T3-L1 adipocytes in the presence or absence of RMR extracts for the measurement of lipolysis and HR-LPL activity—the enzyme critical for lipogenesis in adipocytes. The results showed that cells treated with RMR-W exhibited moderate lipolysis activity accompanied by less cellular TG than the control cells. In contrast, no significant difference in HR-LPL activity was observed between experimental cells and control cells. The lipolytic activity and antihypertrophy effect of RMR were further examined in animal experiments. The addition of 0.4 and 2% RMR to the HF diet significantly enhanced lipolytic activity and attenuated the fat deposition or hypertrophy in perirenal and epididymal adipose tissues.

Furthermore, the body weight gain induced by the HF diet was significantly reversed by RMR treatment. To our knowledge, this is the first study to report the lipolytic activity of RMR. Lipolytic activity contributed to the prevention of fat cell hypertrophy and body weight gain in the HF rats. On the basis of the results, we suggest that the lipolytic components of RMR may be hydrophilic and thus exist mainly in water extracts, which is practical at physiological concentrations. The functional ingredients and the mechanism of action of RMR should be determined in future studies.

In the animal studies in which the HF diet was supplemented with lovastatin and 2% RMR, food/energy consumption decreased significantly, whereas 0.4% RMR treatment also decreased food/energy consumption, but not significantly so ($P>0.05$). It appears that the inhibitory effect of RMR on appetite is related to the monacolin K component.[37]

As expected, RMR supplementation normalized the dyslipidemia induced by the HF diet. Lovastatin is a competitive inhibitor of HMG-CoA reductase, the major rate-limiting enzyme in the cholesterol biosynthesis pathway, which has been introduced to the clinic for the treatment of hypercholesterolemia. Because *Monascus* species produce monacolin K during fermentation, RMR is considered to be a hypocholesterolemic functional food.[5–7] In addition to monacolin K, Hsieh and Tai[8] recently reported that water extracts of RMR decreased the TC level and increased the HDL-C level in rats fed fructose chronically. These results indicate that the benefit of RMR might be due to its content of monacolin K and other ingredients. Furthermore, RMR treatment reduced the hyperinsulinemia induced by the HF diet without affecting the blood glucose level. Previously, Chen and Liu[10] showed that RMR administration exhibited a glucose-lowering effect by enhancing insulin secretion in rats, which resulted from an increase in acetylcholine released by nerve terminals, which in turn stimulated muscarinic M_3 receptors in pancreatic cells. However, an influence of RMR on blood glucose and insulin levels was not seen in a previous study.[8] The discrepancies in results observed between experiments may be due to the different animal models and *Monascus* strains used.

Diet is closely related to the development of obesity; however, certain food components have been reported to exhibit antiobese activity.[48–50] In this study, we explored the effects of RMR on fat accumulation in cellular as well as animal experiments. Our data suggest that the inhibitory effects of RMR on weight gain and the hypertrophy of fat cells were primarily because of lipolytic activity and to a mild antiappetite potency. In addition, proliferation and differentiation in preadipocytes are essential steps to get mature adipocytes, and the inhibition on these processes by the RMR extracts might have the potential to suppress the hyperplasia of fat cells and the related enlargement of adipose tissue. The observed effects of RMR are pleiotropic and likely result from more than one bioactive component. Monacolin K, one of the functional ingredients of RMR, reduced the proliferation in 3T3-L1 preadipocytes and the appetite in HF rats. In brief, this is the first time that the lipolytic activity of RMR has been reported to have the potential to prevent obesity, dyslipidemia and hyperinsulinemia induced by an HF diet.

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