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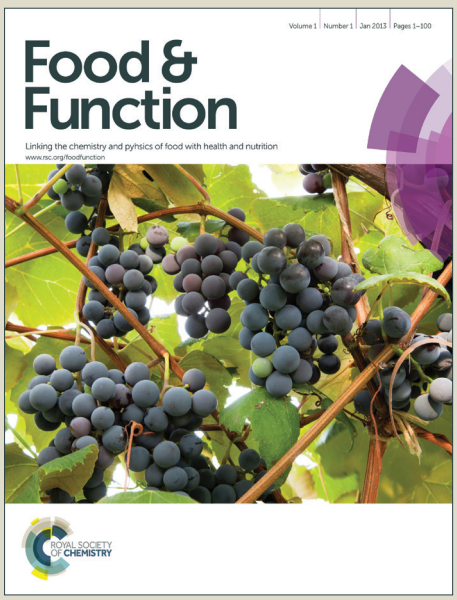

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Regulatory effects of resveratrol on glucose metabolism and T-lymphocyte subsets in the development of high-fat diet-induced obesity in C57BL/6 mice

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Abstract

High-fat diet (HFD)-induced obesity is often associated with immune dysfunction. Resveratrol (trans-3,5,4'-trihydroxystilbene), which has well-founded immunity-related beneficial properties, was used to elucidate the regulatory effect on glucose metabolism and T-lymphocyte subsets in the development of HFD-induced obesity. Resveratrol significantly decreased the body weight and fat masses, being associated with the decrease of plasma leptin, plasma lipid and the release of oxidative stress in HF mice after 26 weeks of feeding. Furthermore, resveratrol decreased the fasting blood glucose and fasting plasma insulin, and increased the $CD3^+CD4^+/CD3^+CD8^+$ subsets percentages and the regulatory T cells (Tregs) production after 13 and 26 weeks of feeding. The results indicated that resveratrol, as an effective supplement for HFD, maintained glucose homeostasis through activating the PI3K and SIRT1 signaling pathway. Meanwhile, resveratrol activated the Nrf2 signaling pathway-mediated antioxidant enzyme expression to alleviate inflammation by protecting against oxidative damage and T-lymphocyte subsets-related chronic inflammatory response in the development of HFD-induced obesity.

1 Introduction

Consumption of high-fat diet (HFD) leads to obesity, insulin resistance, and ultimately type 2 diabetes mellitus,[1] accompanied by the reduced expression of GLUT4 and phosphorylation of AMPKα in skeletal muscle and adipose tissue of C57BL/6 mice.[2] Obesity is often associated with immune dysfunction, which activates numerous inflammatory signaling pathways including IkBα kinases (IKK), c-Jun N-terminal kinases (JNK) and atypical protein kinase C (PKC), in turn inhibiting insulin action.[3] Weight gain during the course of obesity induces chronic inflammation in white adipose tissue (WAT) and liver, leading to the release of proinflammatory cytokine TNF-α[4] that impairs glucose homeostasis by inhibiting insulin action[5,6] Meanwhile, WAT-derived adipokines and chemokines can activate $CD8^+$ T cells in obese states, which then promote macrophage infiltration, thus perpetuating the inflammatory response.[7] Mitogen-stimulated splenocyte proliferation, cytokine production, and antigen-specific antibody in sera were affected in HFD-induced obese mice.[8] Several immune functions and physiological metabolism were changed in mice by high-fat feeding in our previous studies.[9-12] Besides, long-term feeding of HFD induced severe obesity and impaired lymphocytes proliferation in C57BL/6 mice.[13] B cells worsened glucose metabolism through modulation of T cells and production of pathogenic antibodies during high-fat feeding in C57BL/6 mice.[14] Moreover, Tregs in WAT which maintained insulin sensitivity decreased in the course of obesity.[15,16] Thus, lymphocyte subsets are crucial in obesity-related glucose metabolism disorder and chronic inflammatory response.

Resveratrol, a natural polyphenolic compound in grape skins and red wine,[17] promotes vascular endothelial function, anticarcinogenesis and enhances lipid metabolism.[18] The phenolic hydroxyl groups in resveratrol structure are able to scavenge reactive oxygen species (ROS).[19] Resveratrol is also used as a complementary therapeutic for multiple sclerosis that increases the activity of Tregs.[20] Thus, the antioxidative and immunomodulatory properties may primarily contribute to these biological activities.

Obesity is often associated with intensified oxidative stress and immune dysfunction in high-fat fed animals.[21] Our previous study indicated that 0.06% resveratrol functioned more effectively by relieving oxidative stress, inhibiting

expressions of inflammatory genes and increasing Tregs number in HFD-induced obese (DIO) and diet resistant (DR) mice.[10] However, studies concerning the role of resveratrol in the development of HFD-induced obesity and chronic inflammation remain scarce. In this study, we hypothesized that resveratrol could regulate glucose metabolism and T-lymphocyte subsets in the development of HFD-induced obesity. The effects of resveratrol on relief of oxidative stress and the possible relationships with biomarkers associated with metabolic syndrome in the development of HFD-induced obesity were specifically assessed. In addition, the T lymphocytes-activating effects of resveratrol on the $CD3^+CD4^+/CD3^+CD8^+$ subsets percentages were evaluated. Meanwhile, the Tregs production which was involved in obesity as well as chronic inflammation was measured.

2 Materials and methods

2.1 Antibodies and chemicals

Resveratrol was purchased from Shanghai DND Pharm-Technology Co., Inc. (Shanghai, China). PE-Cyanine5-conjugated anti-mouse CD3e mAb (145-2C11 clone; IgG), Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb (GK1.5 clone; IgG2b, κ), PE-conjugated anti-mouse CD8a mAb (53-6.7 clone; IgG2a, κ), PE-Cyanine7-conjugated anti-mouse CD25 mAb (PC61.5 clone; IgG1, λ), and APC-conjugated anti-mouse FoxP3 (FJK-16s clone, IgG2a, κ) and its staining kit were obtained from eBioscience (San Diego, CA).

2.2 Animals

C57BL/6 mice (6-8 week old, female) were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (SLACCAS), All mice were housed under conditions of controlled temperature (23 ± 2 °C) and humidity (60%) with natural light. All experimental animal care and treatment followed the guidelines set up by the Institutional Animal Care and Use Committee of Jiangnan University.

2.3 Experimental design

All the mice were first fed with normal diets for 1wk for acclimatization before the study, and then were randomly assigned to one of the following 3 dietary groups (n=32). Control group (mice were fed with normal diet containing 4.89% fat), HF group (mice were fed with HFD containing 21.45% fat), This HFD has been found to cause significant oxidative stress in C57BL/6 mice in our previous study.[12] HF+R group (mice fed with HFD in supplemention with 0.06% resveratrol, which was mixed with a pelleted diet as used earlier).[22,23] Compositions of the animal diets are listed in Table 1. All mice were allowed free access to the test diets throughout the test period. Their body weights were also monitored weekly. Eight mice for each group were sacrificed at 3, 6, 13 and 26 weeks respectively, overnight food-deprived mice were anaesthetized with diethyl ether inhalation between 8:00 and 10:00 AM. Blood was collected into microcentrifuge tubes containing heparin by orbital vein puncture under anaesthesia and used for fasting blood glucose test and flow cytometry. The animals were euthanized with overdoses of anesthetic. The perirenal fat, periovular fat and liver were washed with normal saline, wiped with filter paper and weighed. Plasma was obtained from blood samples after centrifugation (500g for 10 min at 4°C) and then stored at -20°C until analysis. Spleen was dissected immediately, weighed, and divided into two parts. A portion of the spleen was frozen in liquid nitrogen and stored at -80°C for later use, and the residual was used for flow cytometry and biomarkers of oxidative stress analysis.

2.4 Analysis of fasting blood glucose, fasting plasma insulin, leptin and lipid profile

Fasting blood glucose was measured using reagent strips for a LifeScan SureStep ® Flexx glucose meter (Johnson and Johnson, Rochester, USA). Fasting plasma levels of insulin (Huijia Bioengineering Institute, Xiamen, China), leptin (TSZ Scientific, Framingham, MA, USA) as well as total cholesterol, triacylglycerol, low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol concentrations (Nanjing Jiancheng Bioengineering Institute, Nanjing, PR China) in plasma were measured according to the instructions of the manufacturer. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated (HOMA-IR = fasting blood glucose × fasting plasma insulin/22.5).

2.5 Assessment of antioxidant status in spleen

The spleen tissue was homogenized in a glass-Teflon homogenizer with 50 mM phosphate buffer (PH 7.4) to obtain 1: 9 (w/v 10%) whole homogenate. The supernatant of homogenates was used for the measurement of catalase (CAT), glutathione (GSH), glutathione disulfide (GSSG), total antioxidant capacity (TAC) and malondialdehyde (MDA) according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, PR China).

2.6 Cell preparation

Peripheral blood was resuspended in erythrocyte lysis solution (0.17 mmol/l NH4Cl, 0.01 mmol/l EDTA, 0.1 mol/l Tris, pH 7.3) and then washed twice in RPMI-1640 with 10% fetal calf serum to obtain peripheral blood mononuclear cells (PBMCs). Single-cell suspensions of spleen were prepared according to standard laboratory procedures. In brief, spleen was passed through 70 and 40 mm nylon meshes before erythrocyte lysis. Subsequently, cells were washed several times with PBS and resuspended in RPMI-1640 with 10% fetal calf serum. Viability of the cells was assessed by Trypan blue staining before flow cytometry.

2.7 Immunofluorescence staining and flow cytometry (FCM)

The analysis of $CD3^+CD4^+$ lymphocytes, $CD3^+CD8^+$ lymphocytes and Tregs was performed on PBMCs and splenocytes as described previously.[24,25] Briefly, 50μL of PBMCs and splenocytes were added to falcon tubes (B.D. Biosciences, San Jose, CA) containing different immuno-labeled monoclonal antibodies. For detection of $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes, cells were stained with anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) at room temperature for 30 min in the dark, followed by centrifugation. For Tregs intracellular staining, cells stained with anti-CD4 and anti-CD25 mAbs were fixed with Fix/Perm Buffer (according to the manufacture manual) after being washed and incubated with 1×Permeabilization Buffer for 15 min, then incubated with anti-FoxP3 mAb for 30 min at room temperature according to the instruction offered by the manufacturer. Thereafter the cells were washed and analyzed on FACS Calibur and the data were processed using

FlowJo software (Tree Star, Ashland, OR, USA).

2.8 RNA isolation and gene expression analysis by quantitative real-time reverse transcription PCR

Total RNA of spleen was extracted with Trizol reagent (Invitrogen, USA), and then reverse-transcribed to cDNA according to the manufacturer's instructions (MultiScribe Reverse Transcriptase, Applied Biosystems, Foster City, CA, USA). Platinum Taq polymerase (Invitrogen Life Technologies) and SYBR Green fluorescence (Bioneer Inc. Korea) were used to employ quantitative real-time reverse transcription PCR (qRT-PCR). Target genes were analyzed by qRT-PCR using Applied Biosystems (Foster City, CA) 7900 Fast Real-Time PCR System. A house-keeping gene, β-actin, was used as internal control to normalize the qRT-PCR data. Sequences of the used primers are given in Table 2. The expression level of a gene in a given sample was represented as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta CT = [\Delta CT_{(experimental)}] - [\Delta CT_{(medium)}]$ and $\Delta CT = [CT_{(experimental)}] - [CT_{(housekeeping)}]$.

2.9 Statistical analyses

All results were expressed as means ± SD. Comparisons across groups were performed with SPSS 13 (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) after testing for homogeneity of variance test, and then the means were compared by Tukey's test. Confidence levels for statistical significance were set at $P < 0.05$.

3 Results

3.1 Effect of resveratrol on the body weight, fat masses and plasma leptin of HF mice

There were no significant differences among the body weights of control, HF and HF+R mice after 3 and 6 weeks of feeding (Fig. 1A). The body weights of HF and HF+R mice were significantly higher than that of control mice after 13 weeks of feeding ($P < 0.05$). After 26 weeks of HFD, the body weight of HF mice, which was

33.32% higher than that of control mice, could be significantly decreased by resveratrol supplementation ($P < 0.05$). Similar to the body weight change, the weight of perirenal fat in HF mice was significantly higher ($P < 0.05$, Fig. 1B) than that in control mice after 13 weeks of HFD, while the weight of periovular fat and the fat/body weight (BW) ratio of HF mice increased significantly ($P < 0.05$, Fig. 1C and D) after 6 weeks of HFD. Resveratrol significantly decreased the weights of adipose tissues compared with those of HF mice after 26 weeks of feeding ($P < 0.05$). There were no significant differences among the liver/BW ratio of three groups throughout the dietary protocol (Fig. 1E). Compared with control mice, plasma leptin of HF mice increased significantly after 6 weeks of high-fat feeding, and this trend continued throughout the dietary protocol ($P < 0.05$, Fig. 1F), while resveratrol significantly decreased the plasma leptin of HF mice ($P < 0.05$) after 13 and 26 weeks of feeding.

3.2 Effect of resveratrol on the plasma lipid of HF mice

The total cholesterol of HF mice evidently increased after 6 weeks of HFD and remained thereafter ($P < 0.05$, Table 3), while resveratrol significantly decreased the total cholesterol of HF mice ($P < 0.05$) after 26 weeks of feeding. The plasma triacylglycerol levels of HF mice were not significantly higher than those of control mice after 3 and 6 weeks of high-fat feeding ($P > 0.05$, Table 3), which was significantly increased after 13 weeks of feeding ($P < 0.05$). After 26 weeks of HFD, the plasma triacylglycerol level of HF mice, which was 10.48% higher than that of control mice, was significantly decreased by resveratrol supplementation ($P < 0.05$).

The plasma LDL-cholesterol of HF mice increased after 26 weeks of HFD ($P > 0.05$, Table 3). Resveratrol decreased plasma LDL-cholesterol, especially after 26 weeks of feeding ($P < 0.05$). Moreover, HDL-cholesterol significantly decreased in HF mice after 26 weeks of HFD, which was increased by the supplementation of resveratrol ($P < 0.05$).

3.3 Effects of resveratrol on the glucose metabolism of HF mice

The fasting blood glucose of HF mice increased significantly after 6 weeks of high-fat feeding, and this trend continued throughout the dietary protocol ($P < 0.05$, Fig. 2A). After 6, 13 and 26 weeks of feeding, the fasting blood glucose levels of HF mice were

21.95%, 35.81% and 45.17%, respectively, higher than those of control mice (all $P < 0.05$). Resveratrol significantly decreased the fasting blood glucose compared with those of HF mice after 13 and 26 weeks of feeding ($P < 0.05$). In conjunction with the changes in fasting blood glucose, the fasting plasma insulin level of HF mice were significantly higher than those of the control mice after 6, 13 and 26 weeks of high-fat feeding ($P < 0.05$, Fig. 2B). While, the fasting plasma insulin levels of HF+R mice were 26.13% and 13.46%, respectively, lower than those of HF mice after 13 and 26 weeks of feeding. HOMA-IR of HF mice increased significantly after 6 weeks of HFD ($P < 0.05$, Fig. 2C). Resveratrol decreased it compared with that of HF mice after 13 and 26 weeks of feeding ($P < 0.05$).

3.4 Effects of resveratrol on the oxidative stress of HF mice

There were no significant differences between the splenic CAT activities of HF and control mice after 3 and 6 weeks of feeding (Table 4). After 13 and 26 weeks of feeding, the CAT activities of HF mice which were 36.56% and 52.51%, respectively, lower than those of control mice, were enhanced by resveratrol supplementation ($P < 0.05$). High-fat feeding for 6 weeks significantly decreased GSH/GSSG in the spleen of HF mice compared with that in control mice ($P < 0.05$). There were no significant differences among the GSH/GSSG of three groups after 13 weeks of feeding, while the splenic GSH/GSSG of HF mice was 16.90% lower than that of control mice after 26 weeks of HFD. Resveratrol significantly increased the GSH/GSSG of HF mice after 6 and 26 weeks of feeding ($P < 0.05$). T-AOC significantly decreased and MDA evidently increased in HF mice after 6 weeks of HFD, and remained thereafter ($P < 0.05$, Table 4). Resveratrol increased the T-AOC and decreased MDA compared with those of HF mice throughout the dietary protocol.

3.5 Effects of resveratrol on the $CD3^+CD4^+/CD3^+CD8^+$ percentages of HF mice

The $CD3^+CD4^+/CD3^+CD8^+$ ratios of peripheral blood in HF mice were 23.39% and 12.74% respectively, lower than those of control mice after 13 and 26 weeks of HFD ($P < 0.05$, Fig. 3D). Resveratrol increased the $CD3^+CD4^+/CD3^+CD8^+$ ratio in HF mice, especially after 26 weeks of feeding ($P < 0.05$). The splenic $CD3^+CD4^+/CD3^+CD8^+$ ratio of HF mice significantly decreased after 3 weeks of

high-fat feeding, and this trend continued throughout the dietary protocol ($P < 0.05$, Fig. 3E). Resveratrol elevated the ratio compared with that of HF mice throughout the feeding period.

3.6 Effects of resveratrol on the Tregs production of HF mice

There were no significant differences among Tregs productions in the peripheral blood of the three groups after 3 weeks of feeding (Fig. 4D). After 6, 13 and 26 weeks HFD, the percentages of Tregs in HF mice were 15.65%, 49.25% and 57.93%, respectively, lower than those of control mice ($P < 0.05$, Fig. 4D). Resveratrol significantly increased the Tregs productions in peripheral blood after 13 and 26 weeks of feeding ($P < 0.05$). HFD significantly decreased the splenic Tregs production of HF mice throughout the dietary protocol ($P < 0.05$, Fig. 4E). The Tregs production of HF+R mice increased by 39.32% compared with that of HF mice after 26 weeks of feeding.

3.7 Effects of resveratrol on the expressions of glucose metabolism-related genes in HF mice

High-fat feeding for 26 weeks significantly decreased SIRT1 and GLUT4 expressions compared with those in control mice ($P < 0.05$, Fig. 5A and B). Resveratrol supplementation increased the expressions of SIRT1 and GLUT4 in HF mice, especially after 13 and 26 weeks of feeding ($P < 0.05$).

In HF mice, the expression of PI3K significantly increased after 6 weeks of HFD, which, however, was significantly decreased compared with those of control mice after 13 and 26 weeks of feeding ($P < 0.05$, Fig. 5C). Resveratrol significantly increased the expression of PI3K in HF mice after 26 weeks of feeding ($P < 0.05$). The AKT expressions of HF mice were 33.66% and 39.60%, respectively lower than those of control mice after 13 and 26 weeks of feeding ($P < 0.05$, Fig. 5D). Resveratrol supplementation significantly increased the AKT expression of HF mice after 26 weeks of feeding ($P < 0.05$).

3.8 Effects of resveratrol on the expressions of inflammatory mediators in HF mice

High-fat feeding increased the splenic expression of TNF-α in HF mice throughout the experimental period, especially after 13 and 26 weeks of feeding ($P < 0.05$, Fig. 6A). Resveratrol significantly decreased TNF-α expression after 26 weeks of feeding, which was 32.10% lower than that of HF mice ($P < 0.05$). The expressions of NF-κB and COX-2 in HF mice significantly increased after 6 weeks of HFD, and this trend continued throughout the dietary protocol ($P < 0.05$, Fig. 6B and C). Resveratrol supplementation decreased the NF-κB and COX-2 expressions compared with those of HF mice.

3.9 Effects of resveratrol on the expressions of oxidative stress-related genes in HF mice

GSK-3β expression was significantly increased in HF mice after 13 and 26 weeks of feeding ($P < 0.05$, Fig. 7A), which was significantly decreased by resveratrol supplementation ($P < 0.05$). The Nrf2 expression of HF mice rose significantly after 6 weeks of feeding ($P < 0.05$, Fig. 7B), and then gradually reduced. After 26 weeks of HFD, the Nrf2 expression of HF mice was 34.0% lower than that of control mice, which was significantly elevated by resveratrol supplementation ($P < 0.05$).

High-fat feeding significantly decreased the HO-1 expressions in HF mice as compared with those of control mice after 13 and 26 weeks of feeding ($P < 0.05$, Fig. 7C). The HO-1 expressions of HF+R mice were 49.38%, 21.79% and 43.28%, respectively, higher than those of HF mice after 6, 13 and 26 weeks of feeding. There were no significant differences between the NQO-1 expressions in control and HF mice throughout the dietary protocol (Fig. 7D). While, after 13 and 26 weeks of feeding, the NQO-1 expressions of HF+R mice were 18.69% and 61.45%, respectively, higher than those of HF mice.

4 Discussion

Consumption of HFD is correlated with the onset of obesity and the increase of adipose mass.[26] In our study, the body weight and the weights of adipose tissues in HF mice, which increased significantly since 13 weeks, was significantly reduced by resveratrol after 26 weeks of feeding, revealing that resveratrol exerted potential

anti-obesity effect.[27] There were no significant differences among the liver/BW ratio of three groups in the course of HFD, implying that the performance of obesity was confined to partial tissues (e.g. adipose tissues). Leptin can increase sympathetic activity and energy expenditure as well as exert potent anti-obesity effect.[28] Indeed, plasma leptin levels are elevated in obese rodent[29] and human,[30,31] which is associated with leptin resistance.[30] Being consistent with previous studies,[26,32] plasma leptin in HF mice significantly increased compared with that in control mice since 6 weeks of high-fat feeding, suggesting increased leptin resistance in HF mice. Thus, the decreased plasma leptin in HF+R mice compared with that in HF mice after 13 and 26 weeks of feeding might be responsible, at least in part, for the less weight gain and fat storage in these mice. Leptin levels increase with rising body mass and can stimulate the production of proinflammatory factors TNF-α and IL-6, as well as reactive oxygen species in macrophages and mediate the activation of T cells,[32-34] indicating a relevant role in immunity, not only by maintaining energy homeostasis but also by regulating the function of immune cells. In this study, leptin was involved in the regulation of inflammatory condition,[35] as evidenced by the significant increase of it in HF mice. It is well-known that immune responses require an optimum balance between energy intake and consumption.[36] As suggested by the decreased level of plasma leptin, resveratrol was capable of alleviating inflammation in response to high-fat feeding. In conjunction with the changes in body weight, resveratrol decreased the plasma lipid of HF mice owing to the ameliorating effect on lipid metabolism disorder. The findings are in consistent with a recent study that total cholesterol significantly decreased in response to resveratrol.[37] Resveratrol facilitated the triacylglycerol breakdown triggered by β-adrenergic activation and impaired the lipogenesis in human fat cells,[38] suggesting that this anti-obesity effect of resveratrol was responsible for the relieved fat accumulation.

High-fat feeding increased the fasting blood glucose, fasting plasma insulin and HOMA-IR of HF mice after 6 weeks of feeding, and the trend continued throughout the dietary protocol. Resveratrol decreased the fasting blood glucose, fasting plasma insulin and HOMA-IR of HF mice due to the ameliorating effect on glucose metabolism disorder, which accords with the decreased blood glucose in Zucker obese rats and streptozotocin-induced diabetic rats after resveratrol feeding.[39,40] The decreased fasting plasma insulin in HF+R mice also follow other previously report that resveratrol administration led to increased insulin sensitivity in mice fed a

high-caloric diet.[22] We next study the mechanism underlying the effect of resveratrol on the glucose metabolism in the development of HFD-induced obesity.

SIRT1 is a mammalian deacetylase whose function has been linked to metabolic regulation.[41] Resveratrol, which is SIRT1-dependent,[37,42] leads to a beneficial response to metabolic syndrome. Resveratrol can also attenuate the adipogenesis in 3T3-L1 adipocytes through a SIRT1-dependent pathway and upregulate SIRT1 expressions in differentiated adipocytes, giving rise to lipolysis and mobilization of free fatty acids.[42] In our study, resveratrol administration significantly elevated the SIRT1 expression compared with that of HF mice, which was consistent with a previous study that resveratrol promoted glucose uptake in the presence of insulin in normal and insulin-resistant muscle myocytes through a SIRT1-dependent pathway.[37] The decreased expression of GLUT4 in HF mice suggested that glucose metabolism was destroyed without GLUT4.[43] In addition to the impaired glucose metabolism of skeletal muscle and systemic insulin action, lipid metabolism is also affected by GLUT4 ablation,[44] which is in accordance with the lipid metabolism disorder in HF mice. The increased expression of GLUT4 in HF+R mice resembled that reported previously,[40] inferring that resveratrol augmented glucose uptake. The PI3K signaling pathway plays an important role in regulating obesity and diabetes. Insulin-stimulated glucose transport can be facilitated via PI3K signaling pathway by regulating the expression and activation of GLUT4, PI3K and AKT.[45] In this study, as evidenced by the increased expression of PI3K of HF mice after 6 weeks of HFD, glucose metabolism was activated in the early stage of high-fat feeding. However, the expression plummeted after 13 and 26 weeks of feeding. Meanwhile, resveratrol significantly increased the expressions of PI3K and AKT in HF mice after 26 weeks of feeding, suggesting that resveratrol may play a dominant role in this integrated regulation of glucose homeostasis by improving the PI3K signaling transduction.

It is well known that T lymphocytes play a central role in cell-mediated immunity, such as killing target cells, reacting to specific antigens and producing cytokines. $CD4^+$ and $CD8^+$ T cells are two important subsets that regulate immunity. T cells ($CD3^+$ cells) carry out specialized functions such as cytokine secretion and B cells help through $CD4^+$ and $CD8^+$ T cells.[46] There were significant positive correlations between peripheral $CD3^+CD4^+$/$CD3^+CD8^+$ percentages and host protective cellular or humoral responses in immune compromised conditions such as cancer and tuberculosis. In this study, high-fat feeding induced significant reductions of the

$CD3^+CD4^+/CD3^+CD8^+$ percentages since 13 weeks in peripheral blood and 3 weeks in spleen, suggesting a potential decline of immunity in HF mice. As indicated by the raised proportion of $CD3^+CD4^+/CD3^+CD8^+$ T cells in HF+R mice, resveratrol was beneficial to the immunoregulation induced by high-fat feeding. Tregs, a small subset of T lymphocytes, are one of the body's most crucial defenses against inappropriate immune responses, operating in contexts of autoimmunity, inflammation, infection and tumorigenesis.[47] Tregs also influence the obesity-related inflammatory state in adipose tissue and, thus, insulin resistance.[15] The decreased Tregs production in HF mice herein further demonstrated the aggravation of chronic inflammation by HFD, and resveratrol increased the Tregs production in the peripheral blood and spleen of HF mice, being consistent with the previously proposed role of resveratrol in the regulation of immune functions.[48] It is generally assumed that some inflammatory processes are a consequence of obesity.[4] The adoptive transfer of Tregs can reduce both IKK-β and NF-κB binding activities in HFD-fed mice, thus modulating the inflammatory signaling in steatosis.[49] In the meantime, resveratrol is capable of inhibiting inflammatory adipokines.[50] Hence, whether the effect of resveratrol on the recruitment of Tregs was accompanied by reduced expressions of inflammatory mediators was then analyzed. In this study, resveratrol treatment inhibited the increased expressions of TNF-α, NF-κB and COX-2 induced by HFD throughout the diet period, which further verified its adjuvant role in controlling the inflammatory responses in various tissues.[51,52]

Actually, chronic inflammation functions in the development of insulin resistance and other obesity-related features, commonly recognized as metabolic syndrome manifestations.[53] However, the molecular basis of the origin of this inflammatory condition has not yet been well elucidated. It has been reported that high glucose intake increases intra-cellular ROS in leukocytes and adipocytes.[54,55] ROS also regulate cell death in a variety of cell types.[56] Therefore, the proinflammatory state of obese individuals might be related to chronic excessive nutrient intake.[57] In our study, the antioxidant defenses were weakened in HF mice, confirming that oxidative stress was remarkably increased in high-fat fed animals.[58] Nrf2 signaling is known to be mainly responsible for stimulating of SOD and GSH-px gene expressions and hence exhibits a crucial cellular response to environmental stress.[59] GSK-3β is able to block the protective effect of Nrf2 on the model of excitotoxicity in hippocampus and to inhibit the Nrf2 activity and the phase II response by excluding Nrf2 from the

nucleus.[60] In our study, the increased Nrf2 expression of HF mice after 6 weeks of feeding indicated the activation of Nrf2 that attenuated oxidative stress in the early stage of high-fat feeding. After 13 and 26 weeks of HFD, as evidenced by the increased expression of GSK-3β and the decreased expression of Nrf2 along with Nrf2 translocation-activated HO-1 and NQO1 in HF mice, high-fat feeding suppressed the transcription of key antioxidant genes by triggering the translocation of Nrf2 into the nucleus, thus aggravating the oxidative stress in HF mice. Indeed, the lack of an active Nrf2 signaling pathway in mice can increase inflammation and inflammation-mediated oxidative damage.[61] Therefore, our results are also in accordance with previous study that oxidative stress might trigger a proinflammatory status.[62] Considering the anti-oxidative effect of resveratrol,[63] we specifically assessed the effects of resveratrol on the oxidative stress in spleen and the potential relationships with biomarkers associated with inflammatory condition and metabolic syndrome, and showed that resveratrol increased the battery of antioxidant genes transactivated by Nrf2 to relieve the oxidative stress induced by HFD. Likewise, it has been reported that resveratrol functioned in the activation of Nrf2 as well as in the regulation of antioxidase expression to strengthen its antioxidative activity.[64] Therefore, the beneficial action of resveratrol on HFD-related immunomodulatory effect was antioxidant-mediated.

5 Conclusions

In summary, resveratrol managed to maintain glucose homeostasis in the development of HFD-induced obesity, through activating the PI3K and SIRT1 signaling transduction. Resveratrol also activated the Nrf2-regulated adaptive response to attenuate inflammation by protecting against inflammatory oxidative damage and T lymphocytes-related inflammatory response. Our results expand current understanding of the inflammation in the development of HFD-induced obesity and provide a potential adjuvant candidate for maintaining glucose homeostasis and alleviating inflammation by supplementing resveratrol.

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Figure captions

Fig. 1 Effect of resveratrol on the body weight, fat masses and plasma leptin of HF mice. Data are shown as mean ± SD (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.

Fig. 2 Effect of resveratrol on the fasting blood glucose, fasting plasma insulin and HOMA-IR of HF mice. Data are shown as mean ± SD (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.

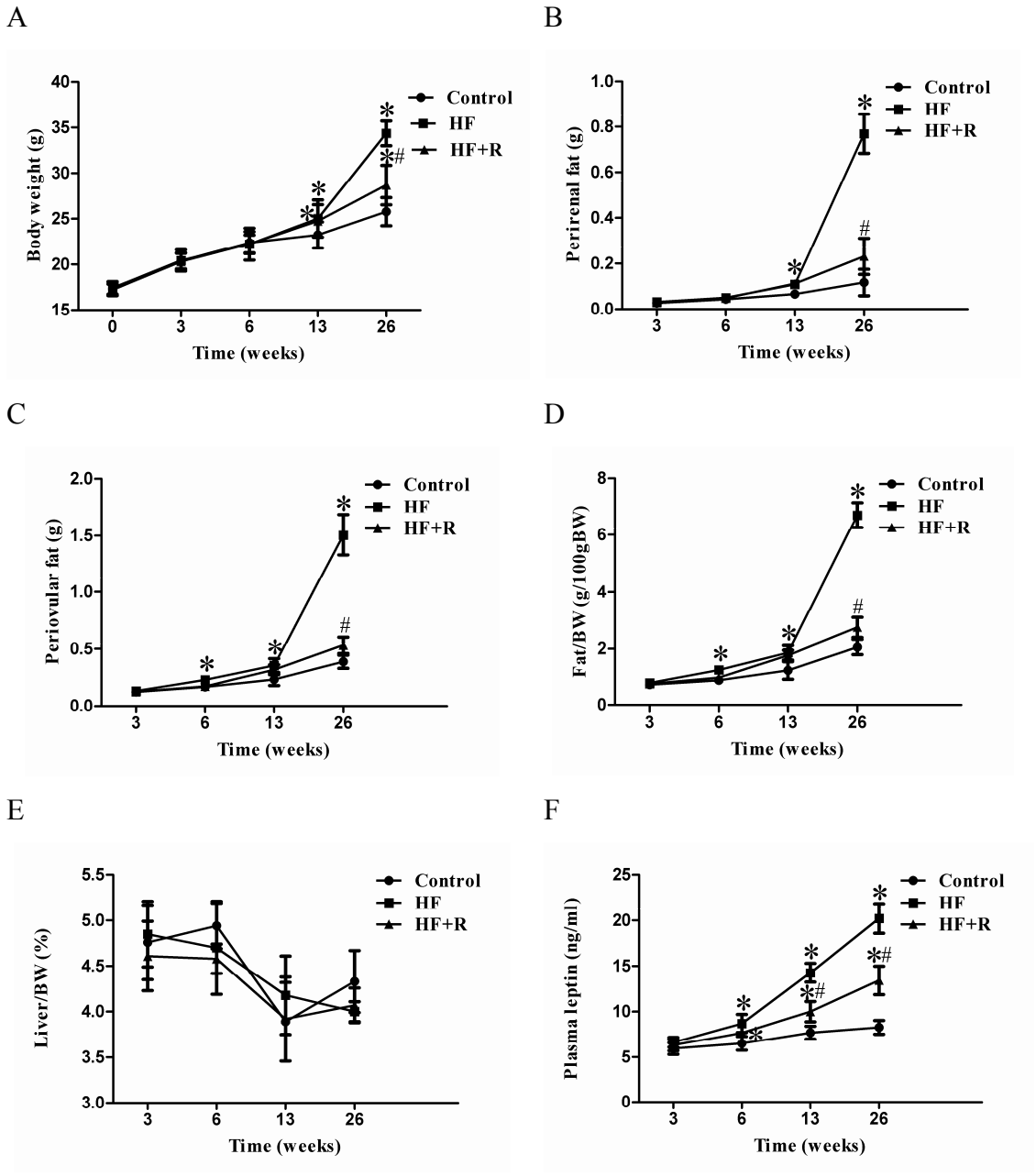
Fig. 3 Flow cytometry detection of $CD3^{+}CD4^{+}$ T cells and $CD3^{+}CD8^{+}$ T cells in peripheral blood and spleen. (A)-(C) Representative flow cytometry dot plots of $CD3^{+}CD4^{+}$ T cells and $CD3^{+}CD8^{+}$ T cells; (D)-(E) Bar graph for the $CD3^{+}CD4^{+}/CD3^{+}CD8^{+}$ percentages in peripheral blood and spleen. Data are shown as mean ± SD (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.

Fig. 4 Flow cytometry detection of $CD4^{+}CD25^{+}$ $FoxP3^{+}$ Tregs in peripheral blood and spleen. (A)-(C) Representative flow cytometry dot plots of $CD4^{+}CD25^{+}$ $FoxP3^{+}$ Tregs; (D)-(E) Bar graph for the Tregs production in peripheral blood and spleen. Data are shown as mean ± SD (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.

Fig. 5 Quantitative real-time reverse transcription PCR detection of SIRT1, GLUT4, PI3K and AKT expressions in spleen. The relative expression level of each sample was calibrated by the comparative threshold cycle method, using β-actin as an endogenous control. Data are expressed as fold changes (mean ± SD), normalized to β-actin mRNA expression, where the values for the control mice were set at 1.0. The analyses were performed in triplicate (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.

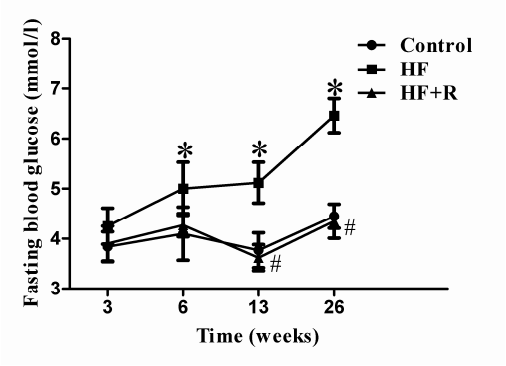
Fig. 6 Quantitative real-time reverse transcription PCR detection of TNF-α, NF-κB and COX-2 expressions in spleen. The relative expression level of each sample was

641 calibrated by the comparative threshold cycle method, using β-actin as an endogenous
642 control. Data are expressed as fold changes (mean ± SD), normalized to β-actin
643 mRNA expression, where the values for the control mice were set at 1.0. The analyses
644 were performed in triplicate (n = 8). $^{*}P < 0.05$ compared with the control mice; $^{\#}P <$
645 0.05 compared with the HF mice.
646
647 **Fig. 7** Quantitative real-time reverse transcription PCR detection of GSK-3β, Nrf2,
648 HO-1 and NQO-1 expressions in spleen. The relative expression level of each sample
649 was calibrated by the comparative threshold cycle method, using β-actin as an
650 endogenous control. Data are expressed as fold changes (mean ± SD), normalized to
651 β-actin mRNA expression, where the values for the control mice were set at 1.0. The
652 analyses were performed in triplicate (n = 8). $^{*}P < 0.05$ compared with the control
653 mice; $^{\#}P < 0.05$ compared with the HF mice.



654 Fig. 1

A



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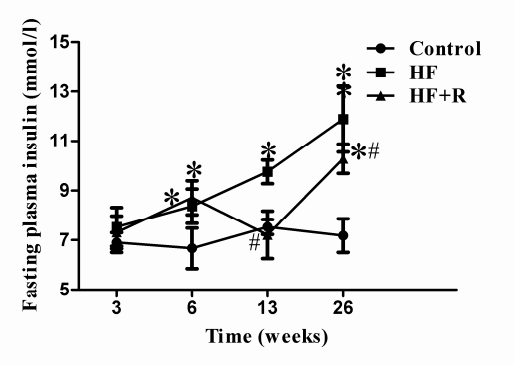

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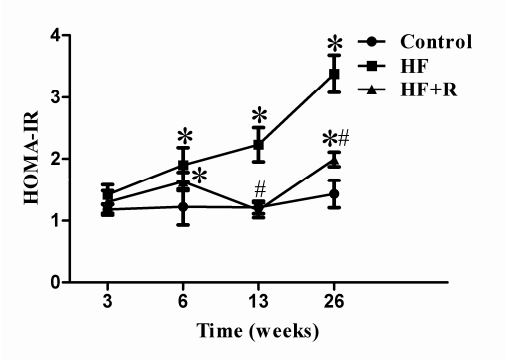
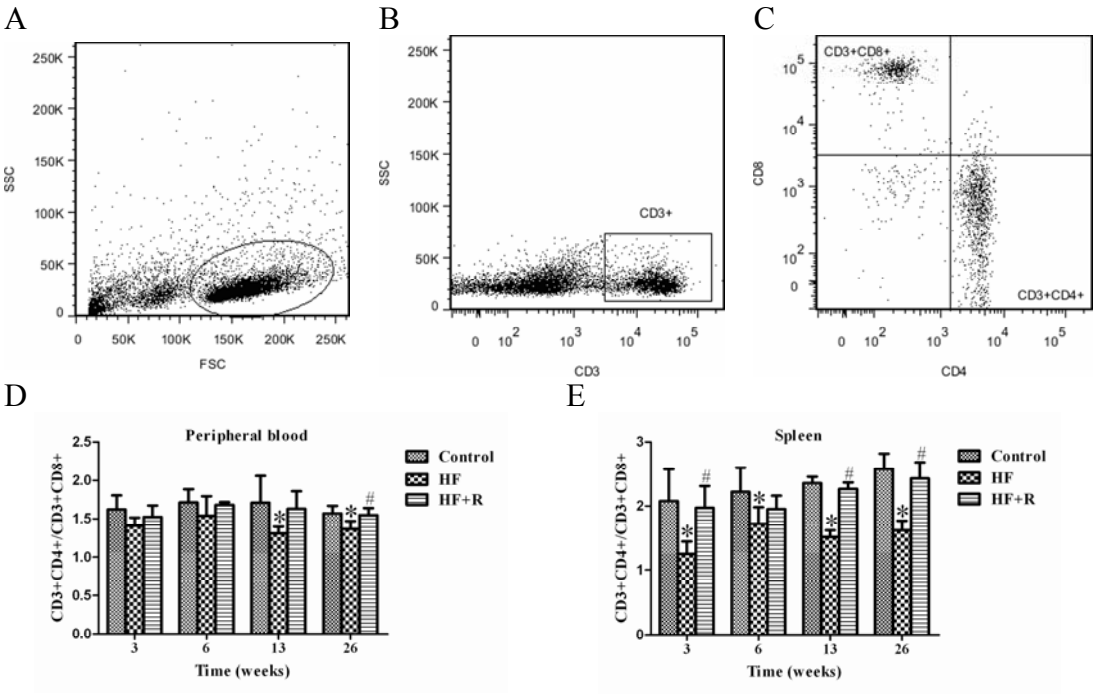
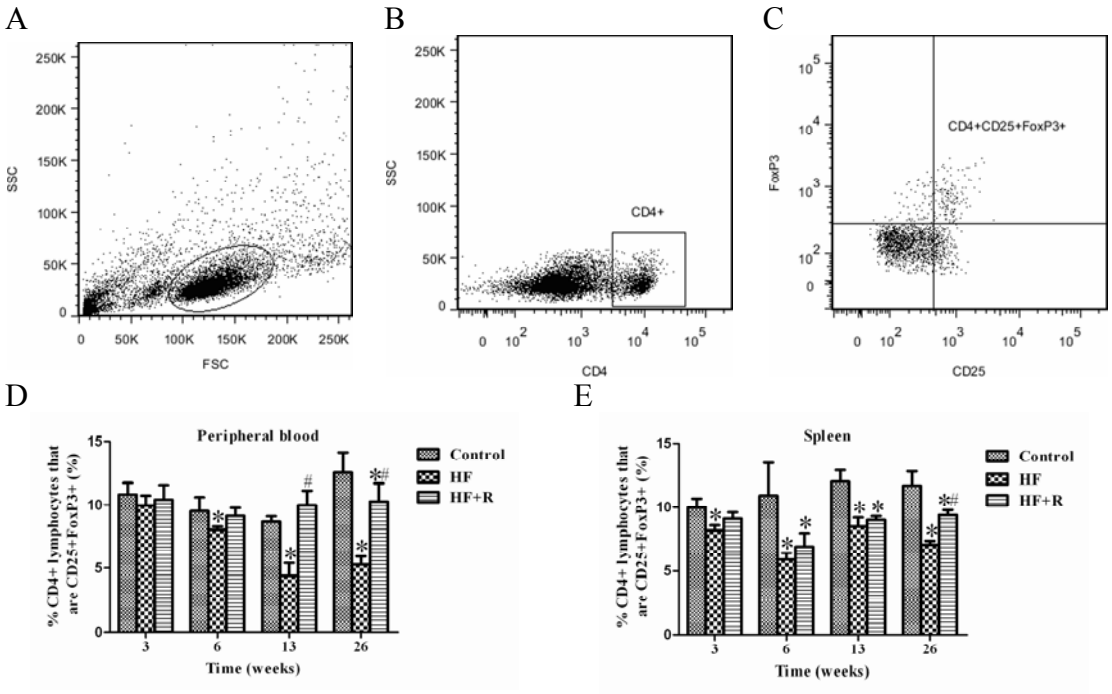


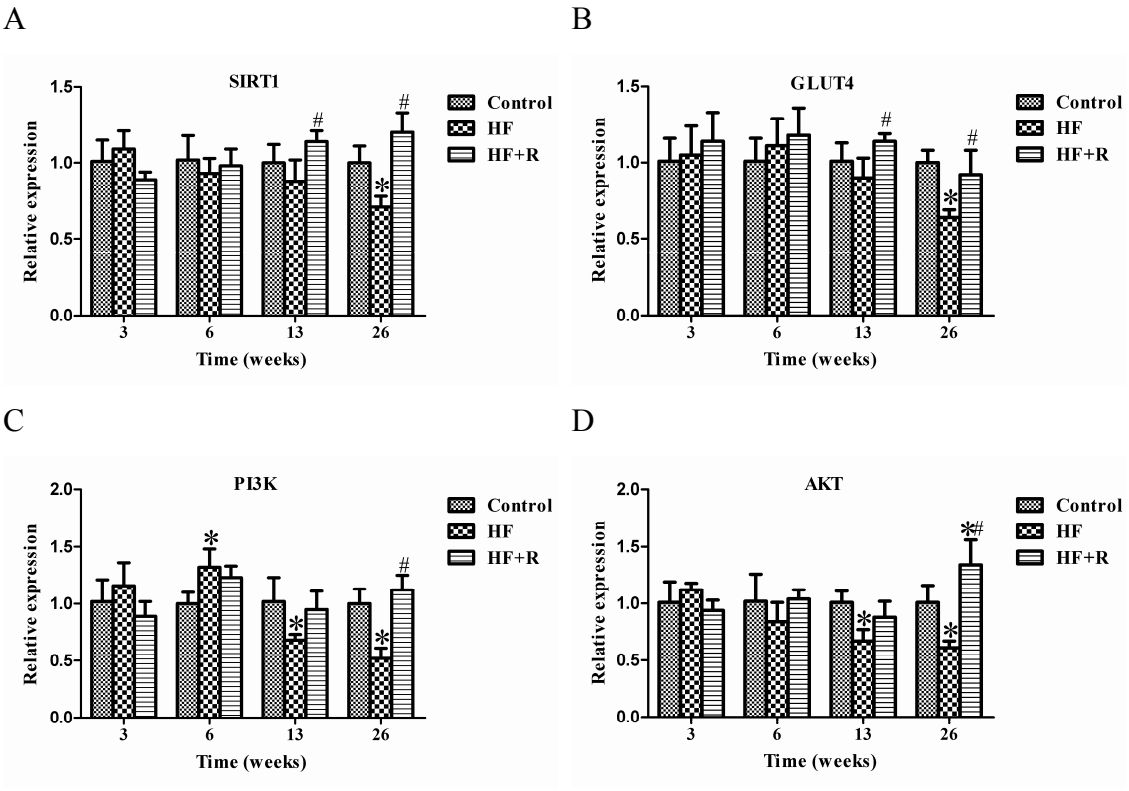
655 Fig. 2



656 Fig. 3

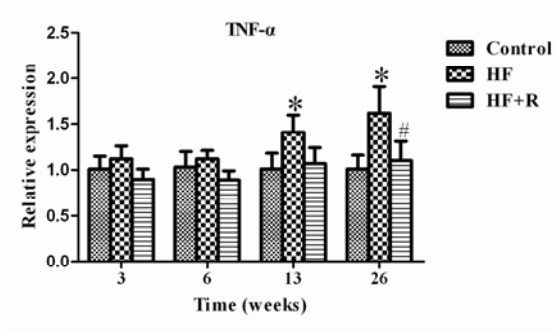


657 Fig. 4



658 Fig. 5

A



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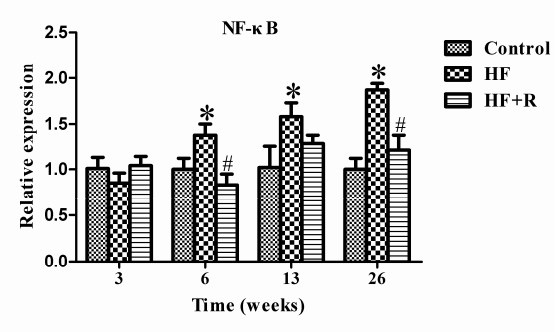

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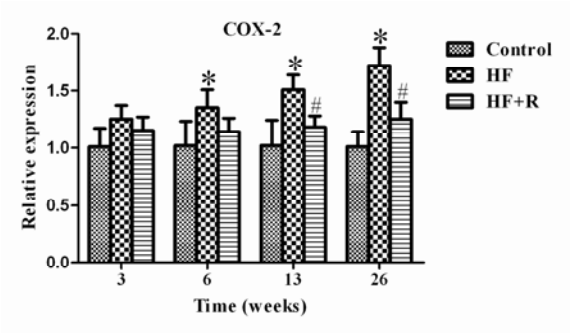

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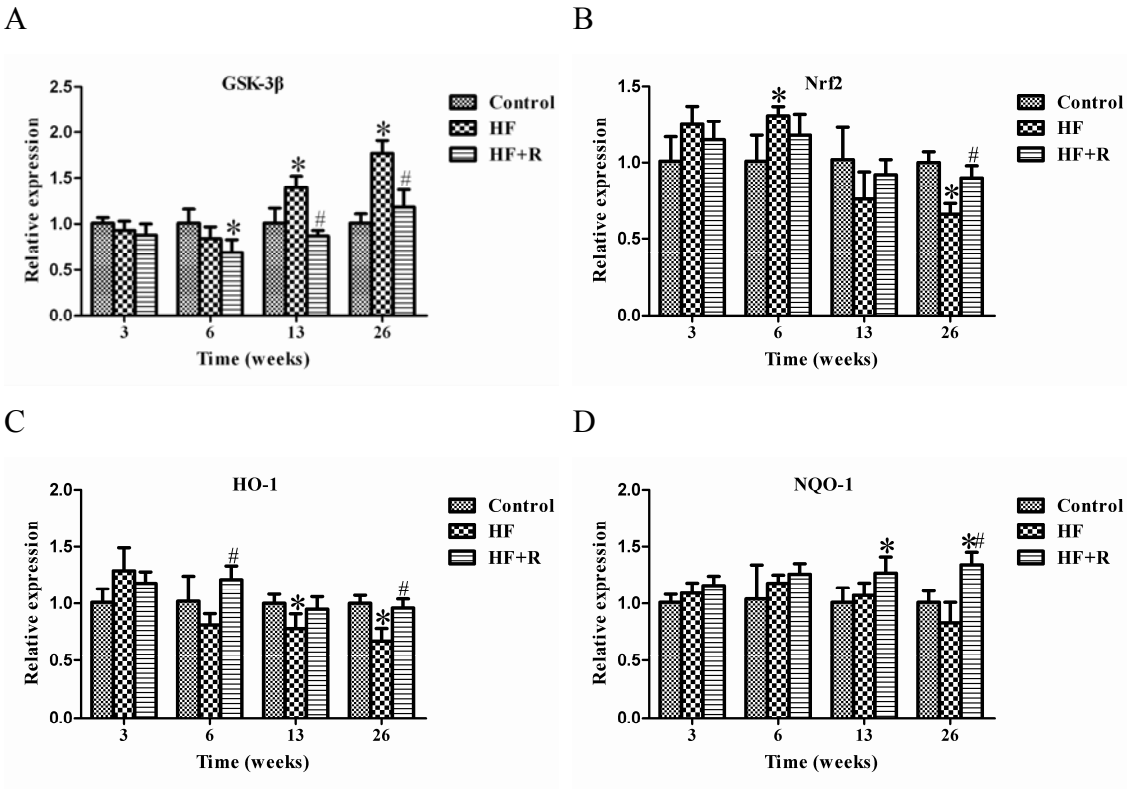

660 Fig. 7

661 **Table 1** Ingredient composition of the diets fed to mice

Ingredient (g/kg diet)	Normal diet	High-fat diet
Cornmeal[a]	484.2	312.4
Soybean meal[b]	243.0	296.5
Wheat flour	150.0	74.8
Corn bran	70.0	84.1
Lard[c]	22.0	196.4
Cholesterol	0	5
$CaHPO_4$	10.0	10.0
$CaCO_3$	13.0	13.0
Lysine	1.2	1.2
Methionine	3.0	3.0
Choline	1.0	1.0
AIN-76 Minerals	0.4	0.4
NaCl	2.0	2.0
AIN-76 Vitamins	0.2	0.2

662 [a] Cornmeal contains 9.2% protein, 73.8% carbohydrate, and 3.5% fat.

663 [b] Soybean meal contains 41.5% protein, 35% carbohydrate, and 5% fat.

664 [c] Lard provides the following (g/100 g lard): 14:0, 2.0; 14:1, 0.3; 15:1, 0.1; 16:0, 26.5;

665 16:1, 3.7; 17:0, 0.5; 17:1, 0.4; 18:0, 12.1; 18:1, 42.5; 18:2(ω-6), 9.8; 18:3(ω-3), 0.7;

666 20:0, 0.2; 20:1, 0.6; 20:4(ω-6), 0.3

667 **Table 2** Oligonucleotide primer design for genes analyzed by quantitative real-time
668 reverse transcription PCR.

Gene	Primer pairs
β-actin	F, GGGTCAGAAGGACTCCTATG R, GTAACAATGCCATGTTCAAT
SIRT1	F, AATAGGGAACCTTTGCCTCATCTAC R, TTGGTGGCAACTCTGATAAATGAAC
GLUT4	F, CCATTCCCTGGTTCATTGTG R, ACGGCAAATAGAAGGAAGAC
PI3K	F, TGCTCCGTAGTGGTAGAC R, GTATGCTAGTGTGACATTGAG
AKT	F, CGGGCACATCAAGATAACG R, CGTGGTCCTGGTTGTAGAAG
TNF-α	F, CTGAACTTCGGGGTGATCGGT R, TCCTCCACTTGGTGGTTTGCTAC
NF-κB	F, AGGCTTCTGGGCCTTATGTG R, TGCTTCTCTCGCCAGGAATAC
COX-2	F, AAAACCTGGTGAACTACGACTGCTA R, CATAGAATAATCCTGGTCGGTTTGA
Gsk-3β	F, TTGGACAAAGGTCTTCCGGCCC R, TGCAGGTGTGTCTCGCCCAT
Nrf2	F, AGCACATCCAGACAGACACCAGT R, TTCAGCGTGGCTGGGGATAT
HO-1	F, ACAGGGTGACAGAAGAGGCTAAGAC R, ATTTTCCTCGGGGCGTCTCT
NQO1	F, GGCGAGAAGAGCCCTGATTG R, GTTCATAGCATAGAGGTCAGATTCG

669 **Table 3** Effect of resveratrol on the plasma lipid in HF mice

	week 3	week 6	week 13	week 26
Total cholesterol (mmol/l)				
Control	1.91±0.28	1.56±0.16	1.08±0.19	1.48±0.18
HF	2.12±0.24	1.94±0.31*	1.50±0.21*	2.07±0.21*
HF+R	2.10±0.21	1.80±0.21	1.77±0.09*	1.64±0.15#
Triacylglycerols (mmol/l)				
Control	2.09±0.11	1.09±0.17	1.10±0.03	3.53±0.24
HF	2.02±0.07	1.25±0.17	1.29±0.07*	3.90±0.21*
HF+R	1.59±0.15#	1.14±0.09	1.28±0.10*	2.82±0.23*#
LDL-cholesterol(mmol/l)				
Control	0.62±0.02	0.70±0.23	0.60±0.12	0.95±0.17
HF	0.74±0.11	0.73±0.12	0.77±0.07	1.08±0.12
HF+R	0.86±0.16	0.42±0.05#	0.70±0.12	0.42±0.09*#
HDL-cholesterol(mmol/l)				
Control	0.84±0.11	1.01±0.11	0.95±0.17	0.95±0.10
HF	0.84±0.15	1.02±0.19	1.03±0.10	0.65±0.07*
HF+R	0.82±0.02	1.03±0.15	1.15±0.11*	0.87±0.08#

670 Data are shown as mean ± SD (n = 8).

671 *$P < 0.05$ compared with the control mice; #$P < 0.05$ compared with the HF mice.

672 **Table 4** Effect of resveratrol on the splenic antioxidant status in HF mice

	week 3	week 6	week 13	week 26
CAT(U/mg prot)				
Control	1.23±0.18	1.91±0.21	1.86±0.11	2.59±0.25
HF	1.35±0.18	2.00±0.39	1.18±0.17*	1.23±0.18*
HF+R	1.39±0.20	2.32±0.24*	1.45±0.24*#	3.16±0.28*#
GSH/GSSG				
Control	2.44±0.33	2.76±0.32	2.25±0.24	2.13±0.21
HF	2.82±0.35	2.38±0.10*	2.23±0.16	1.77±0.11*
HF+R	3.04±0.27*	3.09±0.35#	2.44±0.27	2.40±0.25*#
T-AOC(U/mg prot)				
Control	0.76±0.12	0.46±0.08	0.73±0.13	0.94±0.13
HF	0.74±0.09	0.26±0.02*	0.46±0.05*	0.53±0.07*
HF+R	0.90±0.14#	0.31±0.04*	1.07±0.09*#	0.92±0.11#
MDA(mmol/mg prot)				
Control	2.65±0.21	3.29±0.23	3.05±0.51	4.27±0.58
HF	2.88±0.28	5.27±0.57*	3.90±0.63*	5.78±0.42*
HF+R	2.78±0.18	3.87±0.45#	3.50±0.31	3.56±0.34*#

673 Data are shown as mean ± SD (n = 8).

674 $^{*}P < 0.05$ compared with the control mice; $^{\#}P < 0.05$ compared with the HF mice.