Research Article

Resveratrol ameliorates chronic high altitude exposure-induced oxidative stress and suppresses lipid metabolism alteration in rats

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High altitude affects energy metabolism. Resveratrol is the natural compound that activates sirtuins, which produce beneficial effects on lipid metabolism. The study was conducted to investigate the role of resveratrol in lipid metabolism of rats exposed to a high altitude hypoxia environment. Sprague–Dawley rats were divided into control, resveratrol (30 mg/kg B.W./d) (Res), high altitude hypoxia (HH), and high altitude hypoxia + resveratrol (30 mg/kg B.W./d) (HH+Res) groups. Rats in HH and HH+Res groups were placed in a hypobaric chamber at a stimulated elevation of 4500 m for 30 days. Plasma lipid profile, antioxidant indexes, key enzyme activities, lipid metabolism-related genes, and hypoxia-inducible factors (HIFs) expression were evaluated. Results showed that resveratrol alleviated hypobaric hypoxia-induced enlargement of lung and spleen, and decreased serum malondialdehyde (MDA), plasma high density lipoprotein (HDL) and cholesterol (CHOL) contents, increased liver superoxide dismutase (SOD) activity and Sirtuin 1 (Sirt-1) expression levels, and decreased liver MDA and triglyceride (TG) levels compared to the HH group. Moreover, resveratrol increased fat hormone sensitive lipase (HSL) activity, and suppressed fat Acetyl-CoA carboxylase (ACC), Carnitine palmitoyltransferase (CPT-I) and fatty acid synthetase (FAS) activity. In addition, resveratrol decreased liver CCAAT/enhancer binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), and hypoxia inducible factor-1 alpha and hypoxia inducible factor-2 alpha (HIF-1α and HIF-2α) expression levels compared to the HH group. In conclusion, resveratrol can effectively ameliorate high altitude hypoxia-induced oxidative stress and alteration of lipid metabolism, which is associated with the HIF signaling pathway.

Practical applications: Resveratrol may be a feasible agent for ameliorating high altitude-induced oxidative stress and lipid metabolism dysfunction. Moreover, the HIF-mediated alterations in genes involved in fat metabolism (Srit1, C/EBPα, and PPARγ) may be targets to prevent and treat lipid metabolic disease under a high altitude hypoxia environment.

Keywords: High altitude hypoxia / Hypoxia inducible factors / Lipid metabolism / Resveratrol / Sirt-1

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Abbreviations: ACC, acetyl-CoA carboxylase; **C/EBPα,** CCAAT/enhancer binding protein alpha; **CHOL,** cholesterol; **CPT-I,** carnitine palmitoyltransferase; **FAS,** fatty acid synthetase; **HDL,** high density lipoprotein; **HIF-1α,** hypoxia inducible factor-1α; **HIF-2α,** hypoxia inducible factor-1α; **HSL,** hormone sensitive lipase; **LDL,** low density lipoprotein; **LPL,** lipoprotein lipase; **MDA,** malondialdehyde; **PPARγ,** peroxisome proliferator-activated receptor γ; **ROS,** reactive oxygen species; **SCD-1,** stearoyl-CoA desaturase 1; **SOD,** superoxide dismutase; **Sirt-1,** sirtuin-1; **TG,** triglyceride

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1 Introduction

Millions of people live permanently at high altitudes in North, Central, and South America, East Africa, and Asia. Besides, every year many more people work or travel to high altitude. High-altitude is a special ecological environment, of which the most obvious feature is hypobaric hypoxia. High altitude exposure causes changes in physiological and metabolic processes [1–3]. Acclimatization to high altitude (4300 m) results in decreased reliance on fat as a fuel [4], and selectively decreases key enzymes involved in fat utilization and oxidation in the heart, liver, and certain skeletal muscles [5]. At the usual temperature (22°C), hypoxia attenuated lipid uptake in most tissues, thereby raising plasma TG and LDL cholesterol [6]. Hypoxia-induced regulation of lipid metabolism may carry implications for the risks of diabetes, obesity, and cardiovascular disease. It is imperative to seek for some safe and effective drugs. Although high altitude hypoxia is the most common pattern, studies on this issue are limited. The molecular mechanism for altered patterns of lipid metabolism at high altitude remain to be explained. Understanding the interactions between hypoxia and lipid metabolism is an important requirement for counseling persons living, working or travelling to high altitude.

In recent years, there has been a growing interest in plant compounds with beneficial effects on health [7]. Among these plant compounds, resveratrol has attracted considerable attention as preventive and therapeutic agents. Resveratrol is a natural non-flavonoid polyphenol phytoalexin found in large quantities in the skin of red grapes, berries, red wine, and peanuts [8, 9]. Besides, this plant compound possesses diverse biological and pharmacological effects including cardioprotective effects, anticarcinogenic, antioxidant [10], antidiabetic effects [11], neuroprotective effects. Moreover, resveratrol is an activator of sirtuins [12]. A large number of studies have confirmed that resveratrol decreases lipogenesis [13, 14] and cholesterol level [9], restrict platelet aggression [15], improves metabolism [16]. The main target organs of resveratrol are the liver and kidney, which has been demonstrated in pharmacokinetic and pharmacodynamic studies [17]. Up to now, many studies in the literature have addressed the potential anti-adipogenic effect of resveratrol under normoxic or hypoxia conditions [18–20], the role and mechanisms of resveratrol in lipid metabolism under high altitude hypoxia environment remains unclear.

In this study, we investigated the role of resveratrol in lipid metabolism under a high altitude hypoxia environment through establishing an animal model. We observed the lipid content in liver, plasma lipid profile, antioxidant index in serum and liver, lipid metabolism-related enzyme activity in liver and fat. In addition, we investigated the role of HIFs and the Sirt1/PPARγ/C/EBPα-mediated signal pathway in the modulatory effects of resveratrol on lipid metabolism of rats exposed to a high altitude hypoxia environment.

2 Materials and methods

2.1 Instruments and reagents

The main instruments used in this study were the following: hypobaric chamber (Guizhou Fenglei Aviation Ordnance Co., Ltd, China), ultraviolet spectrophotometer (HITACHI, Japan), fluorescence microscope (Nikon, Japan), CFX96™ Real-Time System (BIO-RAD, USA). The main reagents used in this study were the following: resveratrol (3,5,4′-trihydroxystilbene) with purity of 98.9% (XI'AN APP CHEM-BIO CO., Ltd.), Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), Reverse first strand cDNA synthesis kit (Thermo Scientific, USA), SYBR® Premix Ex Taq™ kit (TaKaRa Biotech Co., Ltd., DALIAN, China), β-actin mouse polyclonal antibody (Santa Cruz, USA), HIF-1α rabbit polyclonal antibody (Santa Cruz, USA); HIF-2α rabbit polyclonal antibody (Abcam, USA), Sirt-1 rabbit polyclonal antibody (Cell Signaling Technol. Inc., USA), C/EBPα rabbit polyclonal antibody(Cell Signaling Technol., Inc., USA), PPARγ rabbit polyclonalantibody (Proteintech Group Inc., USA). Primers used in the study were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.2 Experimental animals

A total of 32 adult male Sprague–Dawley (SD) rats (150 ± 20 g) were purchased from the Experimental Animal Center of College of Medicine, Xi'an Jiaotong University. The disposal of rats is strict comply with the management requirements of experimental animals. All procedures were approved by the Experimental Animal Committee of the Northwestern Polytechnical University (permit number: 20140316).

2.3 Experimental regimen

SD rats were acclimatized for 7 days in stainless steel cages (four rats/cage) at North Western Polytechnical University animal facility (600 m above sea level) at controlled environment conditions (temperature 25 ± 2°C, humidity 50 ± 10%, and a 12 h light/dark cycle) before starting the study. Then, all rats were randomly divided into four groups of eight rats each as follows: a normal control group (Control), a normal control plus resveratrol group (Res), a hypobaric hypoxia group (HH), and a hypobaric hypoxia plus resveratrol group (HH+Res). During the experimental period, rats had free access to water and food. The Control and resveratrol groups were raised in normoxic environment; the other two groups were housed in a hypobaric chamber at a simulated elevation of 4500 m for 30 days. During the period, the rats in Res and HH+Res groups were given resveratrol at 30 mg/kg B.W./d (dissolved in 0.5% sodium carboxymethylcellulose) by gavage daily, while rats in Control and HH groups received the same volume of

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0.5% sodium carboxymethylcellulose. The hypobaric chamber opened 30 min every day from 19:00 to 19:30 at Beijing time for weighing, giving resveratrol, cleaning the cabin and replenishing the animal's food and water.

2.4 Animal observation and sample collection

The spontaneous activity, mental state, and food intake of rats were monitored and recorded every day. After one month exposure to hypobaric hypoxia, the rats were anesthetized with ether, and the abdomen was cut open to collect 5 mL blood from the abdominal aorta. The blood was centrifuged at 3000*g* for 10 min at 4°C, and the serum was separated and stored in Eppendorf tubes. The general condition of rats and overall changes in abdominal cavity were observed. Additionally, the heart, liver, spleen, lungs, and kidney were removed and weighed immediately. About $0.5 \times 0.5 \times 0.5\,cm^3$ of liver was fixed into 4% paraformaldehyde immediately. About 200 mg liver and fat were collected into RNAase-free tubes. All collected tissues were frozen with liquid nitrogen, and then stored at −80°C until needed for analysis.

2.5 Organ index analysis

The organ index was calculated according to the following formula: organ index = wet weight of organ (mg)/body weight (g) .

2.6 Measurement of total SOD activity and MDA levels in liver and serum

Liver tissue was homogenized in 10 volumes (1:10, w/v) of cold normal saline. Homogenates were centrifuged at 5000*g* for 10 min at 4°C. The supernatant was immediately separated and used for the measurements. The total SOD activity, MDA and total protein levels were determined using commercial kits (Nanjing Jiancheng, China) according to the manufacturer's instructions. The SOD activity in liver was adjusted by the protein and expressed as units per milligram of total proteins. The SOD activity in serum was expressed as units per milliliter. MDA level (nmol/mL) = [A (sample) − A (sample blank)]/[A (standard) − A (standard blank)].

2.7 Detection of plasma lipid profiles and liver TG content

Total cholesterol, high density lipoprotein, low density lipoprotein, and triglycerides were estimated in plasma using diagnostic kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). About 100 mg liver was homogenized in 3 volumes (1:3, w/v) of methanol, and then added 6 volumes (1:6, w/v) of chloroform and mixed. After 12 h extraction, 10 μL organic solvent in the lower layer was used to detect the TG content using commercially available TG kits according to the manufacture's recommended protocol. For Oil-Red O staining, the frozen liver was cut into 5–10 μm thick sections, then washed gently with 60% isopropanol, and stained with filtered Oil-Red O solution (60% isopropanol and 40% water) for 15 min. After staining for lipid droplets, the filtered Oil-Red O solution was removed and the plates were rinsed with water and dried. Image of the stained lipid droplets were collected on an Olympus microscope (Tokyo, Japan).

2.8 Detection of ACC, CPT-I, FAS, LPL, and HSL activity in liver and fat

The levels of ACC, CPT-I, FAS, LPL, and HSL in liver and fat were detected using commercially available ELISA kits (DuoSet ELISA development system, R&D, USA) according to the manufacture's recommended protocol. A Synergy HT Multi-Detection Microplate Reader (Bio-Tek) was used to read the optical density at 450 nm, and the concentration of ACC, CPT-I, FAS, LPL, and HSL from the samples was determined using a standard curve.

2.9 RNA extraction and quantitative real-time PCR analysis of Sirt-1, PPARγ, and C/EBPα mRNA level

Total RNA from liver was isolated using the TRIzol method (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacture's protocol. The concentrations and purity of the RNA samples were assessed by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo, USA) at 260 nm and 280 nm. The cDNA was synthesized from the total RNA using PrimeScript™ RT reagent Kit (TaKaRa Bio, Japan) following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with the SYBR® Premix Ex Taq™ II (TaKaRa Bio, Japan) by using a CFX 96™ Real-Time System (Bio-Rad, USA). Primers for rat Sirt-1, PPARγ, C/EBPα, and GAPDH (shown in Table 1) were designed with primer premier 5.0 (Premier Biosoft, USA). GAPDH was used as an endogenous control. The relative gene expression was quantitatively analyzed by the comparative Ct method ($2^{-\triangle\triangle CT}$). Data were normalized to rat GAPDH mRNA levels.

2.10 Western blotting for detection the expression levels of Sirt-1, PPARγ, C/EBPα, HIF-1α, and HIF-2α

Liver tissues (100 mg) were homogenized in 1 mL lysis buffer (Sangon Biotech, China). Total protein was extracted with Protein Extract Kit (Beyotime, China) according to the manufacturer's instructions. Protein concentration was measured via a bicinchoninic acid assay on BioRad protein microassay procedure (Bio-Rad, Hercules, CA). An aliquot containing 30 μg of protein was diluted in loading buffer (loading buffer: sample = 5:1, v/v), heated to 98°C for 10 min, and was separated by 10% sodium dodecyl sulfate-

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Table 1. Primer pairs used in the Real-time PCR reactions

Genes[a)]	Primer sequences[b)]	PCR product (bp)
Sirt-1	[S]5′-TGTTTCCTGTGGGATACCTGA-3′ [AS]5′-TGAAGAATGGTCTTGGGTCTTT-3′	137
PPARγ	[S]5′-CTTTACCACGGTTGATTTCTC-3′ [AS] CAGGCTCTACTTTGATCGCA-3′	140
C/EBPα	[S]5′-AAGGCCAAGAAGTCGGTGGA-3′ [AS]5′-CAGTTCGCGGCTCAGCTGTT-3′	189
GADPH	[S]5′-TGCCACTCAGAAGACTGTGG-3′ [AS]5′-TTCAGCTCTGGGATGACCTT-3′	129

[a)]Sirt-1, sirtuin-1; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha; GADPH, glyceraldehyde-3-phosphate dehydrogenase.
[b)]S, sense primer; AS, anti-sense primer.

polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto a 0.45 μm-pore polyvinylidene difluoride membrane (PVDF, Immuno-Blot, Bio-Rad) at 100 V for 1 h. The membranes were blocked at room temperature for 2 h with 5% fat-free milk in PBS-T (blotting in PBS with 0.1% Tween-20. Sirt-1 (1:1000), PPARγ (1:1000), C/EBPα (1:1000), HIF-1α (1:1000), HIF-2α (1:1000), and β-actin (1:1000) primary antibodies were added, incubated at 4°C overnight. After washing with PBS-T (0.1% BSA), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (diluted 1:3000; Boster Co., Wuhan, China) for 2 h at room temperature. After additional washing, bound conjugates were detected by ECL SuperSignal™ West Pico substrate (Piece, Rockford, IL, USA). Proteins were visualized by exposing the blot to an X-ray film, photographed with a digital camera. The net intensities of individual bands were measured using Quantity One (version 4.6.2). The relative expression levels of the proteins were expressed as the gray value of the target band over the gray value of β-actin in the same sample.

2.11 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and by using Student's *t*-test (SPSS 19.0, Chicago, IL, USA). All data were shown as mean ± standard error of mean (SEM), and differences were considered to be statistically significant at $P < 0.05$.

3 Results

3.1 General conditions including body and main organs

There are no animal deaths during the experiment. The rats in the Control and Res groups were active, energetic, and showed no pathological reaction in the abdominal cavity that was visible to the naked eye. Less activity, depression, and decreased food intake were observed in rats from HH and HH+Res groups at the early stage of high altitude hypoxia exposure. Increase of body weight were observed in groups Control and Res (Fig. 1). However, five days after high altitude hypoxia exposure, the body weight of rats in groups HH and HH+Res was reduced. It then increased again from the sixth day. Overall, the body weights of rats in the normoxic environment were higher than those in the hypobaric chamber. The body weights of rats in group Res increased compared to the Control group, but the body weight of rats in group HH+Res decreased compared to the HH group. Moreover, the eyes of rats exposed to high altitude presented reddish brown. As shown in Table 2, high altitude hypoxia induced the increase of the spleen index ($P < 0.05$) and lung index ($P < 0.01$) compared to the

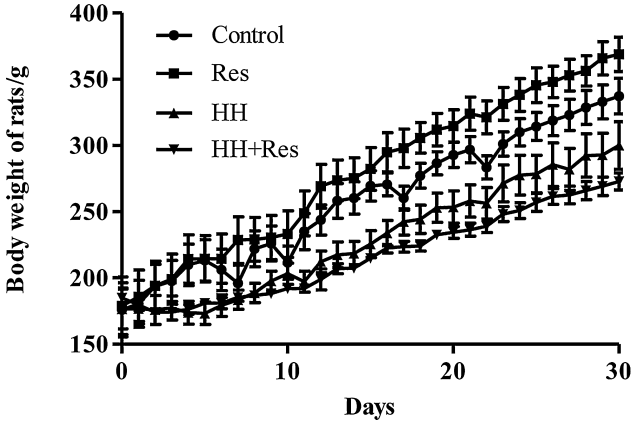

Figure 1. Effects of resveratrol on body weight of rats exposed to hypobaric hypoxia environment. Values are expressed as mean ± SEM. ($n = 8$, each group). Control, Control group; Res, Resveratrol group; HH, High altitude hypoxia group; HH+Res, High altitude hypoxia plus resveratrol group.

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Table 2. Effects of resveratrol on visceral index of rats exposed to hypobaric hypoxia environment

	Visceral index (mg/g)				
Groups	Heart	Liver	Kidney	Lung	Spleen
Control	3.59 ± 0.30	29.03 ± 3.61	7.13 ± 0.68	4.64 ± 0.58	1.84 ± 0.12
Res	3.62 ± 0.39	27.74 ± 1.37	7.28 ± 0.52	4.64 ± 0.40	1.87 ± 0.17
HH	3.84 ± 0.62	30.10 ± 3.35	6.63 ± 0.23	$5.68 \pm 0.36^{**}$	$2.04 \pm 0.10^{*}$
HH+Res	3.98 ± 0.43	28.94 ± 2.23	$7.64 \pm 0.55^{\#\#}$	5.06 ± 0.31	$1.72 \pm 0.20^{\#\#\#}$

Values are expressed as mean ± SEM ($n = 8$, each group). Control, Control group; Res, Resveratrol group; HH, High altitude hypoxia group; HH+Res, High altitude hypoxia plus resveratrol group.
$^{*}P < 0.05$.
$^{**}P < 0.01$ vs. Control.
$^{\#\#}P < 0.01$.
$^{\#\#\#}P < 0.001$ vs. HH.

Control group. The kidney index was significantly increased ($P < 0.01$), and spleen index was significantly decreased ($P < 0.001$) in group HH+Res compared to the HH group.

3.2 Total SOD activity and MDA levels in serum and liver

As shown in Table 3, compared to the Control group, high altitude hypoxia stress significantly reduced the liver SOD activity ($P < 0.01$), and increased MDA level in liver ($P < 0.01$) and serum ($P < 0.01$) in the HH group. On the condition of high altitude hypoxia, administration of resveratrol significantly increased liver SOD activity ($P < 0.001$) and inhibited the MDA levels in liver ($P < 0.01$) and serum ($P < 0.05$) when compared with the HH group.

3.3 CHOL, TG, HDL, and LDL contents in plasma and TG content in liver

TG content in plasma was lower ($P < 0.05$) in HH group compared to the Control group. HDL content in plasma was lower ($P < 0.05$) in Res group compared to the Control group (Table 3). When compared to the HH group, HDL content ($P < 0.01$) and CHOL content ($P < 0.05$) in plasma was lower in HH+Res group. TG content in group HH was higher ($P < 0.001$) compared to the Control group. Liver TG content in group HH+Res was lower ($P < 0.05$) compared to that in group HH (Fig. 2).

3.4 ACC, CPT-I, FAS, LPL, HSL activities in liver and fat

Liver ACC ($P < 0.05$) and HSL ($P < 0.001$) activity in the HH group were decreased compared to those in the Control group (Table 3). Liver FAS ($P < 0.05$) and LPL ($P < 0.01$) activity in the HH group were higher than those in the Control group. ACC and CPT-I activity of fat were significantly increased in the HH group ($P < 0.001$, $P < 0.001$) and in the Res group ($P < 0.001$, $P < 0.05$) compared to those in the Control group. FAS ($P < 0.001$) and HSL ($P < 0.001$) activity of liver were decreased in HH group compared to the Control group. Moreover, HSL ($P < 0.01$) activity of fat in the Res group was lower than that in Control group. ACC ($P < 0.001$), CPT-I ($P < 0.001$), and FAS ($P < 0.001$) activity of fat in the HH+Res group were lower than those in the HH group. However, HSL activity of fat in the HH+Res group was increased compared to that in the HH group ($P < 0.001$).

3.5 The mRNA levels of Sirt-1, PPARγ, and C/EBPα in liver

From Fig. 3, we found that the liver Sirt-1 mRNA level were decreased in the group HH compared to that in group Control ($P < 0.001$), and increased in the group HH+Res HH ($P < 0.001$) compared to that in group HH. The liver PPARγ and C/EBPα mRNA levels in groups Res ($P < 0.001$) and HH ($P < 0.01$) were significantly increased compared to that in group Control. However, the liver PPARγ mRNA level in group HH+Res was significantly lower than that in group HH ($P < 0.001$).

3.6 Sirt-1, PPARγ, C/EBPα, HIF-1α, and HIF-2α protein expression in liver

Western blot analysis (Fig. 3) showed that the expression level of Sirt-1 and C/EBPα in group HH were lower than that in group Control ($P < 0.01$). The expression level of HIF-1α and HIF-2α were higher than that in group Control ($P < 0.001$). Moreover, the expression level of C/EBPα ($P < 0.05$), HIF-1α ($P < 0.001$), and HIF-2α ($P < 0.001$) in group HH+Res were lower than that in group HH. In addition, treatment with resveratrol under high altitude hypoxia significantly improved the expression level of Sirt1

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Table 3. Effects of resveratrol on antioxidant indexes, lipid profiles and key enzymes involved in fat metabolism in rats exposed to high altitude environment

	Groups			
Items	Control	Res	HH	HH+Res
Serum				
SOD (U/mL)	521.20 ± 17.80	537.90 ± 25.98	466.50 ± 43.50	469.50 ± 43.80
MDA (nmol/mL)	3.33 ± 0.07	3.75 ± 0.19	4.24 ± 0.21*	3.16 ± 0.31$^{\#}$
Liver				
SOD (U/mgprot)	184.67 ± 7.33	171.41 ± 2.63	155.11 ± 5.12**	197.70 ± 8.51$^{\#\#\#}$
MDA (nmol/mgpro)	1.07 ± 0.09	1.05 ± 0.03	1.63 ± 0.20**	1.19 ± 0.05$^{\#\#}$
Plasma				
TG (mmol/L)	0.43 ± 0.02	0.37 ± 0.02	0.31 ± 0.02*	0.35 ± 0.03
CHOL (mmol/L)	1.63 ± 0.07	1.50 ± 0.06	1.48 ± 0.10	1.17 ± 0.05$^{\#}$
LDL (mmol/L)	0.59 ± 0.03	0.48 ± 0.03	0.64 ± 0.02	0.52 ± 0.08
HDL (mmol/L)	0.87 ± 0.04	0.77 ± 0.03*	0.72 ± 0.04	0.54 ± 0.02$^{\#\#}$
Liver				
ACC (U/L)	941.48 ± 28.53	932.11 ± 27.36	793.96 ± 48.51*	836.81 ± 41.25
CPT-I (U/L)	197.01 ± 8.21	219.50 ± 16.95	232.54 ± 14.54	191.14 ± 2.59
FAS (U/l)	48.12 ± 3.34	50.64 ± 2.04	57.08 ± 1.22*	59.32 ± 2.19
LPL (U/L)	506.99 ± 33.46	581.01 ± 25.78	648.99 ± 21.42**	598.98 ± 12.36
HSL (U/L)	633.28 ± 22.39	635.45 ± 23.27	395.53 ± 17.95***	459.41 ± 6.44
Adipose tissue				
ACC (U/L)	940.03 ± 27.66	1180.20 ± 20.97***	1355.30 ± 4.88***	890.12 ± 11.28$^{\#\#\#}$
CPT-I (U/L)	225.06 ± 1.98	241.48 ± 4.09*	268.18 ± 1.38***	185.00 ± 7.50$^{\#\#\#}$
FAS (U/L)	71.86 ± 0.97	69.31 ± 0.51	60.52 ± 0.27***	47.47 ± 1.70$^{\#\#\#}$
LPL (U/L)	615.66 ± 26.76	561.76 ± 15.46	563.56 ± 11.98	583.47 ± 29.69
HSL (U/L)	626.13 ± 4.09	522.24 ± 10.13**	453.12 ± 12.45***	697.51 ± 22.86$^{\#\#\#}$

Values are expressed as mean ± SEM ($n = 8$, each group). Control, Control group; Res, Resveratrol group; HH, High altitude hypoxia group; HH+Res, High altitude hypoxia plus resveratrol group. SOD, superoxide dismutase; MDA, malondialdehyde; CHOL, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; ACC, Acetyl-CoA carboxylase; CPT-I, carnitine palmitoyltransferase; FAS, fatty acid synthetase; LPL, lipoprotein lipase; HSL, hormone sensitive lipase.
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$ vs. Control.
$^{\#}P < 0.05$.
$^{\#\#}P < 0.01$.
$^{\#\#\#}P < 0.001$ vs. HH.

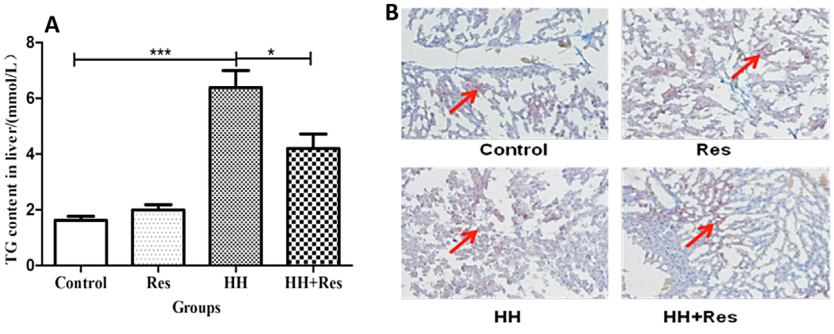

Figure 2. (A) Liver triglyceride (TG) content. (B) Typical photographs of liver histology. Liver sections from rats in the Control, Res, HH, and HH+Res group were analyzed by Oil Red O staining. The magnification of photomicrographs was 200×. Values are expressed as mean ± SEM ($n = 8$, each group). $^{*}P < 0.05$, $^{***}P < 0.001$. Control, Control group; Res, Resveratrol group; HH, High altitude hypoxia group; HH+Res, High altitude hypoxia plus resveratrol group.

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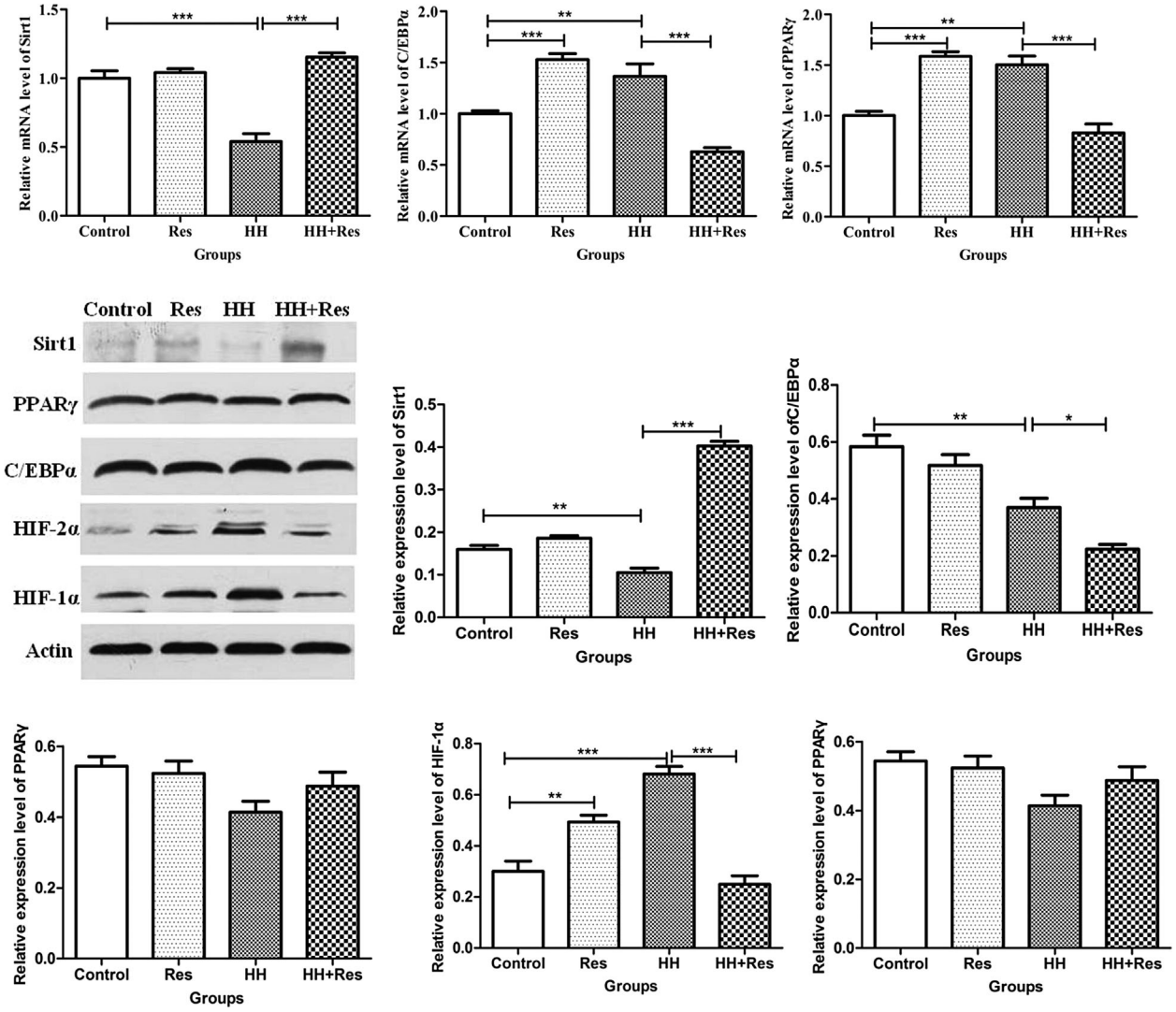

Figure 3. Sirt-1, C/EBPα, PPARγ, HIF-1α, and HIF-2α expression levels in the liver of rats exposed to hypobaric hypoxia environment. Values are expressed as mean ± SEM ($n = 8$, each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Control, Control group; Res, Resveratrol group; HH, High altitude hypoxia group; HH+Res, High altitude hypoxia plus resveratrol group. Sirt-1, sirtuin-1; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha.

when compared with the HH group ($P < 0.001$). However, high altitude exposure and (or) resveratrol treatment did not affect the expression levels of PPARγ ($P > 0.05$).

4 Discussion

Lipid metabolism under high altitude-hypoxia remains poorly understood. In this study, we investigated the role and mechanism of resveratrol in lipid metabolism in rats when exposed to high altitude hypoxia environment for 30 days. Marked body weight loss was observed after high altitude hypoxia exposure, which was consistent with previous studies [3, 21, 22]. Moreover, less food intake was observed in our current and previous experiments. For human, sea level residents lost weight during their acute exposure to high altitude hypoxia [23]. Weight loss under any sort of hypoxia exposure may be largely due to anorexia through a leptin-related effect, increased lipolysis, and increased metabolic rate [24, 25]. Administration of resveratrol induced the increase of body weight under normal environment, which was controversial with other researches [26, 27]. The conclusion of resveratrol with beneficial effect of reducing body weight in literatures was drawn based on the studies in obese animals or animals fed a high-calorie diet. Maybe in normal physiology, resveratrol

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mainly exerts antioxidant activity. Surprisingly, under hypobaric hypoxia environment, resveratrol presented the opposite effect. The potential mechanism of resveratrol regulating lipid metabolism may be different under normoxic and hypoxia.

TG increase in mice within 6 h of hypoxic exposure [28]. Acute exposure to moderate altitude causes a decrease in TG and total cholesterol/HDL ratio as well as an increase in HDL [29]. In the present study, significant decrease in serum TG and significant increase in liver TG were observed after high altitude exposure for 30 days. Alterations of lipid profile may be closely correlated directly with the severity of hypoxia and exposure duration. An increase in HDL was observed on day 7 of high altitude exposure [30]. Changes in lipid profile may be due to the following reasons: the fat mobilization was reduced due to hypoxia over time; the increased catecholamine levels during hypoxia led to reduced lipolysis and a depot of intracellular fat [31]. In addition, most of the decrease in TG was attributable to a decline in VLDL. Recent study suggests that the underlying mechanism involved in the lipid alterations after chronic hypobaric hypoxia seems to be mediated by the up regulation of stearoyl-CoA desaturase 1 (SCD-1) expression [22]. Resveratrol reduced serum HDL and CHOL contents, and liver TG content of rats exposed to high altitude hypoxia. Previous study confirmed that resveratrol reduced total cholesterol and triacylglycerol plasma level [32]. Published studies demonstrate that resveratrol has an anti-adipogenic effect which involves a down-regulation of C/EBPα and PPARγ [33]. Moreover, different metabolic pathways involved in triacylglycerol metabolism in white adipose tissue have been shown to be targets for resveratrol [25].

As high altitude hypoxia produced reactive oxygen species (ROS) directly, the counter balancing effect of the antioxidant enzymes is lost [3, 34]. Oxidative damage and other related diseases occurred. Our results indicate that chronic exposure to high altitude cause the decrease of SOD and increase of MDA (the end product of lipid peroxidation). Lipid peroxidation was found to be increased in liver and muscle following hypoxia as a consequence of oxidative stress [35]. Previous investigation indicated that 6 months of intermittent exposure to high altitude (4000 m) resulted in a decreased activity and protein content of mitochondrial SOD in skeletal muscle of rats [36]. Further study confirmed that a simulated altitude of 5500 m decreased Mn-SOD levels in the liver and lungs by day 21, and increased MDA levels in serum, liver, heart, and kidney [37]. In the current study, resveratrol treatment increased the liver SOD levels and decreased MDA levels in liver and serum of rats exposed to high altitude hypoxia. These results suggest that antioxidant supplementation can attenuate and prevent the oxidative damage associated with high altitude. The protective effects of resveratrol against hypobaric hypoxia stress may be attributable to its ability to modulate cellular redox state [38–40].

High altitude hypoxia exposure is a stressor to an organism and may cause gene expression to change. These genes are involved in lipid metabolism [41]. Our results indicated that exposure to high altitude hypoxia caused the increase of ACC and CPT-I activity in fat and LPL activity in liver, and the decrease of FAS activity in fat. These results suggested that fat mobilization was increased in adipose tissue and TG storage was increased in liver. Hypoxic exercise training decreased fatty acid oxidation in the liver by reducing the CPT1A protein expression and enhancing the protein expression ratio of FAS/CPT1A, while increasing the fatty acid oxidation in gastrocnemius by increasing the CPT1A mRNA level [42]. CPT-I activity was significantly reduced in the heart and extensor digitorum longus, but not altered in the soleusmuscle or the liver [43]. The myocardium of rats exposed to high altitude demonstrated a reduced ability to utilize fatty acids after prolonged exposure [44]. By 30 days after exposure, the expression levels of CPT-I and PPARα returned to the control level [45]. Resveratrol treatment suppressed the fat ACC, CPT-I and FAS activity, and improved the fat HSL activity of rats exposed to high altitude hypoxia. It is interesting to note that resveratrol displayed opposite effects on lipid metabolism-related enzymes under normal and high altitude hypoxia environment. These results were consistent with its effect on body weight of rats. After resveratrol treatment, rising of ACC and CPT-I accelerated the fatty acid oxidation in fat under normal environment. The above data suggest that modulatory effect of resveratrol on lipid metabolism may be through selectively regulating key enzymes in fat utilization and oxidation in different tissues. Moreover, this effect may be closely associated with the physiological status.

Members of the HIF pathway orchestrate molecular responses during hypoxic stress through a complex series of cellular metabolites [46]. HIF-1α and HIF-2α are involved in hypoxia-induced lipid accumulation in hepatocytes via reducing PGC-1α mediated fatty acid β-oxidation [47]. In the study, resveratrol treatment reversed the up regulation of HIF-1α, HIF-2α, C/EBPα, and PPARγ expression, and down regulation of Sirt1 caused by exposure to high altitude hypoxia. Fat anabolic pathways are up regulated in hypoxia, this is mediated at least in part by up regulation of PPARγ [48]. The activation of PPARγ regulates lipid metabolism genes, such as LPL, Acetyl-coenzyme synthetases (ACSs), and FAS [49, 50]. The Sirt1 inhibits the expression of PPARγ to inhibit fat storage and promote fat mobilization [51]. C/EBPα works in the rear stages of adipocytes, and can activate a series of specific gene expression. Resveratrol was reported to act as a natural activator of Sirt1 deacetylase, a modulator of cellular metabolism [52]. A previous study showed that Sirt1 exerts protective effects on metabolic damage induced by a high-fat diet [53]. Recent study suggested that the activation of AMPK by resveratrol and the subsequent effect on lipid metabolism is at least partially dependent on Sirt1 [54]. Therefore, those results suggest that resveratrol may exert beneficial effects on lipid metabolic states through HIFs-mediated alterations in key genes

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involved in fat metabolism after chronic altitude exposure. The exact mechanism needs further investigation.

5 Conclusions

In summary, our results showed that resveratrol can effectively ameliorate chronic high altitude hypoxia-induced oxidative stress and lipid metabolism alteration in rats. The mechanism may be related to its antioxidant properties, modulation of lipid metabolism-related gene expression, and selectively regulating key enzymes involved in fat utilization and oxidation in different tissues. HIF-1α and HIF-2α could mediate the regulation of resveratrol on lipid metabolism under hypobaric hypoxia. Moreover, under a normoxic and high altitude hypoxia environments, resveratrol displays different modulatory effects on lipid metabolism. Findings from the present and other studies suggest that pharmacological modulators of the redox pathway, such as resveratrol, may be feasible agents for ameliorating high altitude-induced oxidative stress and lipid metabolism dysfunction. However, further studies are needed to provide data on the use of resveratrol in cases involving high altitude exposure.

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References

[1] Salama, S. A., Omar, H. A., Maghrabi, I. A., AlSaeed, M. S. et al., Iron supplementation at high altitudes induces inflammation and oxidative injury to lung tissues in rats. *Toxicol. Appl. Pharmacol.* 2014, *274*, 1–6.

[2] Morash, A. J., Kotwica, A. O., Murray, A. J., Tissue-specific changes in fatty acid oxidation in hypoxic heart and skeletal muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2013, *305*, R534–R541.

[3] Xu, C. L., Sun, R., Qiao, X. J., Xu, C. C. et al., Protective effect of glutamine on intestinal injury and bacterial community in rats exposed to hypobaric hypoxia environment. *World J. Gastroenterol.* 2014, *20*, 4662–4674.

[4] Roberts, A. C., Butterfield, G. E., Cymerman., A., Reeves, J. T. et al., Acclimatization to 4,300-m altitude decreases reliance on fat as a substrate. *J. Appl. Physiol.* 1996, *81*, 1762–1771.

[5] Kennedy, S. L., Stanley, W. C., Panchal, A. R., Mazzeo, R. S., Alterations in enzymes involved in fat metabolism after acute and chronic altitude exposure. *J. Appl. Physiol.* 2001, *90*, 17–22.

[6] Jun, J. C., Shin M., Yao, Q. L., Devera, R. et al., Thermoneutrality modifies the impact of hypoxia on lipid metabolism. *Am. J. Physiol. Endocrinol. Metab.* 2013, *304*, E424–E435.

[7] Pal, S., Ho, N., Santos, C., Dubois, P. et al., Red wine polyphenolics increase LDL receptor expression and activity and suppress the secretion of ApoB100 from human HepG2 cells. *J. Nutr.* 2003, *133*, 700–706.

[8] Cho, I. J., Ahn, J. Y., Kim, S., Choi, M. S. et al., Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters. *Biochem. Biophys. Res. Commun.* 2008, *367*, 190–194.

[9] Kim, W. K., Kang, N. E., Kim, M. H., Ha, A. W., Peanut sprout ethanol extract inhibits the adipocyte proliferation, differentiation, and matrix metalloproteinases activities in mouse fibroblast 3T3-L1 preadipocytes. *Nutr. Res. Pract.* 2013, *7*, 160–165.

[10] Su, H. C., Hung, L. M., Chen, J. K., Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. *Am. J. Physiol. Endocrinol. Metab.* 2006, *290*, E1339–E1346.

[11] Zhang, J., Chen, L., Zheng, J., Zeng, T. et al., The protective effect of resveratrol on islet insulin secretion and morphology in mice on a high-fat diet. *Diabetes Res. Clin. Pract.* 2012, *97*, 474–482.

[12] Gertz, M., Nguyen, G. T. T., Fischer, F., Suenkel, B. et al., A molecular mechanism for direct sirtuin activation by resveratrol. *PLoS ONE* 2012, *7*, e49761.

[13] Alberdi, G., Rodríguez, V. M., Macarulla, M. T., Miranda, J. et al., Hepatic lipid metabolic pathways modified by resveratrol in rats fed an obesogenic diet. *Nutrition* 2013, *29*, 562–567.

[14] Lasa, A., Schweiger, M., Kotzbeck, P., Churruca, I. et al., Resveratrol regulates lipolysis via adipose triglyceride lipase. *J. Nutr. Biochem.* 2012, *23*, 379–384.

[15] Das, S., Alagappan, V. K., Bagchi, D., Sharma, H. S. et al., Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: A potential mechanism for resveratrol preconditioning of the heart. *Vascul. Pharmacol.* 2005, *42*, 281–289.

[16] Momken, I., Stevens, L., Bergouignan, A., Desplanches, D. et al., Resveratrol prevents the wasting disorders of mechanical unloading by acting as a physical exercise mimetic in the rat. *FASEB J.* 2011, *25*, 3646–3660.

[17] Colom, H., Alfaras, I., Maijó, M., Juan, M. E. et al., Population pharmacokinetic modeling of trans- resveratrol and its glucuronide and sulfate conjugates after oral and intravenous administration in rats. *Pharmaceutical Res.* 2011, *28*, 1606–1621.

[18] Hu, P., Zhao, L., Chen, J., Physiologically achievable doses of resveratrol enhance 3T3-L1 apipocyte differentiation. *Eur. J. Nutr.* 2015, *54*, 569–579.

[19] Konings, E., Timmers, S., Boekschoten, M. V., Goossens, G. H. et al., The effects of 30 days resveratrol supplementation on adipose tissue morphology and gene expression pattern in obese men. *Int. J. Obesity* 2014, *38*, 470–473.

[20] Powell, R. D., Swet, J. H., Kennedy, K. L., Huyhh, T. T. et al., Resveratrol attenuates hypoxic injury in a primary hepatocyte model of hemeorrhagic shock and resuscitation. *J. Trauma Acute Care Surg.* 2014, *76*, 409–417.

[21] Xu, C. L., Dong, C., Xu, C. C., Han, T. T. et al., Effect of iron supplementation on the expression of hypoxia-inducible factor

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and antioxidant status in rats exposed to high-altitude hypoxia environment. *Biol. Trace Elem. Res.* 2014, *162*, 142–152.

[22] Siques, P., Brito, J., Naveas, N., Pulido, R. et al., Plasma and liver lipid profiles in rats exposed to chronic hypobaric hypoxia: Changes in metabolic pathways. *High Alt. Med. Biol.* 2014, *15*, 388–395.

[23] Ge, R. L., Wood, H., Yang, H. H., Liu, Y. N. et al., The body weight loss during acute exposure to high-altitude hypoxia in sea level residents. *Acta Physiol. Sin.* 2010, *62*, 541–546.

[24] Tschop, M., Strasburger, C. J., Hartmann, G., Biollaz, J. et al., Raised leptin concentrations at high altitude associated with loss of appetite. *Lancet* 1998, *352*, 1119–1120.

[25] Sierra-Johnson, J., Romero-Corral, A., Somers, V. K., Johnson, B. D., Effect of altitude on leptin levels, does it go up or down? *J. Appl. Physiol.* 2008, *105*, 1684–1685.

[26] Aquirre, L., Fernandez-Quintela, A., Arias, N., Portillo, M. P., Resveratrol: Anti-obesity mechanisms of action. *Molecules* 2014, *14*, 18632–18655.

[27] Szkudelska, K., Szkudelski, T., Resveratrol, obesity and diabetes. *Eur. J. Pharmacol.* 2010, *635*, 1–8.

[28] Jun, J. C., Shin, M. K., Yao, Q., Bevans-Fonti, S. et al., Acute hypoxia induces hypertriglyceridemia by decreasing plasma triglyceride clearance in mice. *Am. J. Physiol. Endocrinol. Metab.* 2012, *303*, E377–E388.

[29] Stowhas, A. C., Latshang, T. D., Lo Cascio, C.M. et al., Effects of acute exposure to moderate altitude on vascular function, metabolism and systemic inflammation. *PLoS ONE* 2013, *8*, e70081.

[30] Vats, P., Ray, K., Majumadar, D., Amitabh, et al., Changes in cardiovascular functions, lipid profile, and body composition at high altitude in two different ethnic groups. *High Alt. Med. Biol.* 2013, *14*, 45–52.

[31] Strobel, G., Neureither, M., Bartsch, P., Effect of acute mild hypoxia during exercise on plasma free and sulphoconjugated catecholamines. *Eur. J. Appl. Physiol. Occup. Physiol.* 1996, *73*, 82–87.

[32] Oliveira Andrade, J. M., Paraíso, A. F., Macedo de Oliveira, M. V., de Barros Lima Martins, A. M. E. et al., Resveratrol attenuates hepatic steatosis in high-fat fed mice by decreasing lipogenesis and inflammation. *Nutrition* 2014, *30*, 915–919.

[33] Kwon, J. Y., Seo, S. G., Yue, S., Cheng, J. X. et al., An inhibitory effect of resveratrol in the mitotic clonal expansion and insulin signaling pathway in the early phase of adipogenesis. *Nutr. Res.* 2012, *32*, 607–616.

[34] Seth, V., Banerjee, B. D., Chakravorty, A. K., Lipid peroxidation, free radical scavenging enzymes, and glutathione redox system in blood of rats exposed to propoxur. *Pestic. Biochem. Phys.* 2001, *71*, 133–139.

[35] Singh, S. N., Vats, P., Kumria, M. M. L., Ranganathan, S. et al., Effect of high altitude (7,620 m) exposure on glutathione and related metabolism in rats. *Eur. J. App. Physiol.* 2001, *84*, 233–237.

[36] Radak, Z., Lee, K., Choi, W., Sunoo, S. et al., Oxidative stress induced by intermittent exposure at a simulated altitude of 4000 m decreases mitochondrial superoxide dismutase content in soleus muscle of rats. *Eur. J. Appl. Physiol. Occup. Physiol.* 1994, *69*, 392–395.

[37] Nakanishi, K., Tajima, F., Nakamura, A., Yagura, S. et al., Antioxidant system in hypobaric-hypoxia. *J. Physiol.* 1995, *489*, 869–876.

[38] Zheng, J., Chen, L., Zhang, H., Hu, X. et al., Resveratrol improves insulin resistance of catch-up growth by increasing mitochondrial complexes and antioxidant function in skeletal muscle. *Metabolism* 2012, *61*, 954–965.

[39] Olas, B., Nowak, P., Kolodziejczyk, J., Ponczek, M. et al., Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. *J. Nutr. Biochem.* 2006, *17*, 96–102.

[40] Robb, E. L., Stuart, J. A., Resveratrol interacts with estrogen receptor-β to inhibit cell replicative growth and enhance stress resistance by upregulating mitochondrial superoxide dismutase. *Free Radic. Biol. Med.* 2011, *50*, 821–831.

[41] Bazel, M. M., Schlauch, K., Hayes, J. P., Gene expression of the liver in response to chronic hypoxia. *Physiol. Genomics* 2010, *41*, 275–288.

[42] Lu, Y. L., Jing, W., Feng, L. S., Zhang, L. et al., Effects of hypoxic exercise training on microRNA expression and lipid metabolism in obese rat livers. *Zhejiang Univ. Sci. B* 2014, *15*, 820–829.

[43] Kennedy, S. L., Stanley, W. C., Panchal, A. R., Mazzeo, R. S., Alterations in enzymes involved in fat metabolism after acute and chronic altitude exposure. *J. Appl. Physiol.* 2001, *90*, 17–22.

[44] Bass, A., Ostadal, B., Prochazka, J., Pelough, V. et al., Intermittent high altitude-induced changes in energy metabolism in the rat myocardium and their reversibility. *Physiol. Bohemoslov.* 1989, *38*, 155–161.

[45] Ni, Q., Shao, Y., Wang, Y. Z., Jing, Y. H. et al., Impact of high altitude on the hepatic fatty acid oxidation and synthesis in rats. *Biochem. Biophys. Res. Commun.* 2014, *446*, 574–579.

[46] Majmundar, A. J., Wong, W. J., Simon, M. C., Hypoxia-inducible factors and the response to hypoxic stress. *Mol. Cell* 2010, *40*, 294–309.

[47] Liu, Y., Ma, Z., Zhao, C., Wang, Y. et al., HIF-1α and HIF-2α are critically involved in hypoxia-induced lipid accumulation in hepatocytes through reducing PGC-1α-mediated fatty acid β-oxidation. *Toxicol. Lett.* 2014, *226*, 117–123.

[48] Krishnan, J., Suter, M., Windak, R., Krebs, T. et al., Activation of a HIF alpha-PPARgamma axis underlies the integration of glycolytic and lipid anabolic pathways in pathologic cardiac hypertrophy. *Cell Metab.* 2009, *9*, 512–524.

[49] Contreras, A. V., Torres, N., Tovar, A. R., PPAR-α as a Key Nutritional and environmental sensor for metabolic adaptation. *Adv. Nutr.* 2013, *4*, 439–452.

[50] Ahn, K. O., Lim, S. W., Yang, H. J., Li, C. et al., Induction of PPAR gamma mRNA and protein expression by rosiglitazone in chronic cyclosporine nephropathy in the rat. *Yonsei Med. J.* 2007, *48*, 308–316.

[51] Chen, Y. C., Kao, T. H., Tseng, C. Y., Chang, W. T. et al., Methanolic extract of black garlic ameliorates diet-induced obesity via regulating adipogenesis, adipokine biosynthesis, and lipolysis. *J. Funct. Foods* 2014, *9*, 98–108.

[52] Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W. et al., Small molecule activators of surtuins extend Saccharomyces cerevisiae lifespan. *Nature* 2003, *425*, 191–196.

[53] Pfluger, P. T., Herranz, S., Velasco-Miguel, S., Serrano, M. et al., Sirt1 protects against high-fat diet-induced metabolic damage. *Proc. Natl. Acad. Sci.* 2008, *105*, 9793–9798.

[54] Hou, X., Xu, S., Maitland-Toolan, K. A., Sirt1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J. Biol. Chem.* 2008, *283*, 20015–20026.

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