

# Effects of *Lycium barbarum* polysaccharides on oxidative stress in hyperlipidemic mice following chronic composite psychological stress intervention

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**Abstract.** Chronic composite psychological stress intervention is the accumulation of factors which may induce psychological stress, including food deprivation, water deprivation and swimming in cold water. Approximately 40% of cases of atherosclerosis are associated with chronic composite psychological stress. The aim of the present study was to explore the effects of *Lycium barbarum* polysaccharides (LBP) on blood lipid levels and oxidative stress in hyperlipidemic mice, following chronic composite psychological stress. A hyperlipidemic mouse model was generated, and the mice were subjected to chronic composite psychological stress and treated with LBP for 30 days. After 30 days the triglyceride (TG) and total cholesterol (TC) levels were measured in the serum, and the mRNA expression levels of cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) were determined in the liver, in order to observe any changes to lipid metabolism. The levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were measured in the liver to evaluate the effects of LBP on oxidative stress. The blood serum levels of interleukin-6 (IL-6) and heat shock protein 70 (HSP-70) were measured to evaluate the extent of the aortic inflammatory response, and to determine the protective effects of LBP. The levels of TG, TC, MDA and IL-6 were significantly higher in the mice subjected to chronic composite psychological stress (HS), as compared with the mice treated with LBP alone (HL), or treated with LBP and subjected to stress (HLS). In addition, SOD and HSP-70 levels, and the mRNA expression levels of CYP7A1 were significantly lower in the HS group, as compared

with that in the HL and HLS groups. These results suggest that chronic composite psychological stress may promote the occurrence and development of atherosclerosis, by inducing the aortic inflammatory response and lipid peroxidation. Furthermore, treatment with LBP significantly inhibited oxidative stress and the aortic inflammatory response.

## Introduction

Psychological stress is defined as a process by which the body adapts and responds to threats or challenges that the individual perceives as stressful, resulting in cognitive-induced psychological and physiological changes (1). The psychological stress model is divided into three categories according to the types of psychological stress factors, which produced three different models, as described by Yalcin *et al* (2), including the social stress model, the conflict stress model and the life state stress model. As a single method may not fully simulate the multi-stress factor social environment of human beings, resistance to these factor is easily achievable; therefore, Isingrini *et al* (3) proposed the chronic psychological stress animal model, which combined the three types of psychological stress models in order to produce a realistic simulation of human psychological stress.

Oxidative stress refers to the excessive cellular accumulation of oxygen free radicals and associated metabolites, which results in tissue damage. Oxidative stress is caused by the generation of reactive oxygen species (ROS) and/or their ineffective clearance (4).

Chronic composite psychological stress has recently been suggested as a harmful stimulus that may result in the accumulation of ROS, resulting in damage to tissue and cells (5). It is well known that psychological stress is associated with an increased risk of atherosclerosis (AS) (6), one study reported that ~40% of cases of atherosclerosis were associated with chronic composite psychological stress (7). A recent study identified a correlation between the onset of AS and lipid peroxidation (8). Therefore, further investigation regarding the effects of chronic composite psychological stress on lipid metabolism, oxidative stress and its prevention is of particular importance.

*Lycium barbarum* polysaccharides (LBP) are effective components that can be extracted from Solanaceae plants,

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Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Gene name	Primer sequences	GenBank
7 $\alpha$ -hydroxylase	Forward primer: 5'-CTTCATCACAAACTCCCTGTCA-3' Reverse primer: 5'-TCACTTGGGTCTATGCTTCTGT-3'	NM_007824.2
$\beta$ -actin	Forward primer: 5'-ATATCGCTGCGCTGGTCGTC-3' Reverse primer: 5'-AGGATGGCGTGAGGGAGAGC-3'	

which are found in Ningxia province (China) and have been used in Tradition Chinese Medicine for their anti-fatigue, anti-aging and anti-tumor effects as well as for liver protection and the reduction of blood glucose and lipid levels (9). LBP has previously been identified as an enhancer of the immune system, which was able to improve and strengthen the antioxidant ability of mice suffering from a reduced immune function caused by exercise-induced stress (10). However, it remains to be elucidated whether LBP can affect the blood lipid levels of hyperlipidemic mice, and strengthen their resistance to oxidative stress following chronic composite psychological stress. Therefore, the present study aimed to provide a scientific basis for the development and use of Solanaceae plants, and various other antioxidant tonifying drugs, through investigating the effects of LBP on blood lipid levels and oxidative stress following chronic composite psychological stress in hyperlipidemic mice.

## Materials and methods

**Animals.** A total of 45 male KM mice (32.6 $\pm$ 2.8 g; 6-8 weeks old), which were provided by the Animal Center of Ningxia Medical University, were housed, nine per cage, at 24.0 $\pm$ 0.2°C in a humidified atmosphere (54 $\pm$ 5%). Mice were fed with standard rodent animal feed according to the SPF level and kept under a natural circadian rhythm, with natural lighting and regular ventilation. The experimental procedures of the present study were approved by the Animal Ethics Committee of Ningxia Medical University and Use Committee (Yinchuan, China), in accordance with the guidelines of the Council of the Physiological Society of China.

Following a one week adaptation period, all of the mice were randomly divided into five groups (n=9/group): Normal control group (NC); hyperlipidemia group (H); hyperlipidemia + LBP group (HL); hyperlipidemia + chronic composite psychological stress group (HS); and hyperlipidemia + LBP + chronic composite psychological stress group (HLS). The NC group was fed a normal diet, whereas the other groups were continuously fed a high cholesterol diet (0.5% sodium deoxycholate, 1.25% cholesterol; Jiangsu Matheson Biological Pharmaceutical Co. Ltd., Jinagsu, China). Once the hyperlipidemic mouse model had been successfully established after four weeks, the HL and HLS groups received 80 mg/kg/day LBP (Ningxia Qiyuan Pharmaceutical Co., Ltd, Ningxia, China) by oral gavage for 30 days. The NC, H and HS groups received the same volume of isotonic saline solution by oral gavage for 30 days. The dose of LBP was selected on the basis of preliminary experiments, which demonstrated it to be a safe and effective dose with no toxic side effects in mice.

**Stress protocol.** The HS and HLS mice were subjected to chronic composite psychological stress after the establishment of the hyperlipidemic model. The stress scheme was slightly modified from that previously described by Isingrini *et al* (3), and consisted of the following: Food deprivation for 12 h; water deprivation for 12 h; swimming in cold water (10°C) for 6 min; tail clamping for 1 min; wet feeding (200 ml water was added to the sawdust at the bottom of the cage) for 12 h; sound stimulation (110 dB) for 1 h; high level oscillation (200 r/min) for 45 min; extended daytime for 12 h; inclined feeding (mouse cage without sawdust, tilted 45 degrees) for 12 h; and restraint stress. Between one and two stressors were randomly applied every day for 30 days, the occurrence of which the mice could not anticipate.

**Samples collection.** Immediately after application of the final stressor, the mice were administered 20% urethane (Wuhan Dahua Weiye Medicine Chemical Co. Ltd, Wuhan, China) sacrificed by collection of a lethal dose of blood from the orbital vein. Afterwards, the chest cavity was rapidly opened, and the thoracic aorta was carefully isolated and placed into 4% formaldehyde solution. The livers were also harvested and ~30 mg liver tissue was immediately frozen in liquid nitrogen and stored at -80°C for determination of the mRNA expression levels of cholesterol 7 $\alpha$ -hydroxylase (CYP7A1). The remaining liver tissue was frozen at -80°C and used to measure the levels of malondialdehyde (MDA) and superoxide dismutase (SOD). Blood was collected from the orbital vein in heparinized tubes (Cangzhou Rehabilitation Medical Supplies Co, Ltd, Hebei, China) and centrifuged at 1,700 x g for 10 min (at 4°C) to obtain serum. The serum was frozen at -80°C and subsequently used to measure the levels of triglyceride (TG), total cholesterol (TC), interleukin-6 (IL-6) and heat shock protein 70 (HSP-70).

**Measurement of SOD and MDA levels in the liver.** In order to determine whether LBP was able to reduce the degree of chronic composite psychological stress-induced oxidative stress, oxidative stress indices were measured. The levels of SOD and MDA were determined using commercially available kits, Total Superoxide Dismutase Test kit and Malondialdehyde Test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

**Measurement of TC, TG, IL-6 and HSP-70 levels in the serum.** The serum levels of TC, TG, IL-6 and HSP-70 were detected using commercially available sandwich ELISA kits, including the Mouse Interleukin-6 ELISA kit, Mouse Heat-Shock Protein-70 ELISA kit, Mouse Cholesterol Total ELISA kit and Mouse Triglyceride ELISA kit (Yonghui

Biotechnology, Beijing, China). Absorbance was read at 450 nm using a Bio-Rad 680 microplate reader (Beijing Yonghui Biological Technology Co., Ltd., Beijing, China) following the addition of stop solution within 15 min. The quantity of TC in the serum was estimated from a calibration curve, which ranged between 0.3 and 7 nmol/l. The quantity of TG in the serum was estimated from a calibration curve, which ranged between 1 and 30 nmol/l. The quantity of IL-6 in the plasma was estimated from a calibration curve, which ranged between 8 and 40 pg/ml. The quantity of HSP-70 in the plasma was estimated from a calibration curve, which ranged between 10 and 400 pg/ml.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RNA was extracted from the liver, which was homogenized using a Teflon hammer head, using the RNA AxyPrep Pure RNA Isolation kit (Axygen Scientific, Inc., Union City, CA, USA), according to the manufacturer's instructions. The purity of the RNA was determined using a 721 spectrophotometer (Third Shanghai Analytical Instrument Factory, Shanghai, China) by determining the ratio of absorbance between 260 and 280 nm, and by 1% agarose gel electrophoresis. The RNA samples were analyzed using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). cDNA was synthesized using a Reverse Transcription kit (TransGen Biotechnology, Beijing), according to the manufacturer's instructions. qPCR was performed using a Quantitect SYBR® Green PCR kit (TransGen Biotech Co., Ltd., Beijing, China). The primers used for the PCR are presented in Table I,  $\beta$ -actin was used as an internal control. Initial denaturation was performed at 94°C for 5 min; and PCR amplification was performed by 36 cycles of: 94°C for 30 sec (denaturation), 60°C for 30 sec (primer annealing) and 72°C for 30 sec (extension).

Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method (11,12).

**Morphological observation of the thoracic aorta.** In order to determine whether chronic composite psychological stress had an effect on the morphology of the thoracic aorta in mice, and whether LBP exhibited a protective effect, the morphological changes to the thoracic aorta were observed by hematoxylin and eosin staining using an Olympus DP71 microscope (Olympus, Jiangsu, China).

**Statistical analysis.** The data are expressed as the mean  $\pm$  standard deviation. The statistical analyses were performed using SPSS for Windows version 17.0 software (SPSS Inc., Chicago, IL, USA). Comparisons between the means of numerous samples were evaluated by analysis of variance, and comparisons between the means of two samples were evaluated by a least significant difference t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of LBP on TG and TC levels following chronic composite psychological stress in hyperlipidemic mice.** Disorders of lipid metabolism are closely associated with the occurrence and development of AS (13). Therefore, the present study aimed to

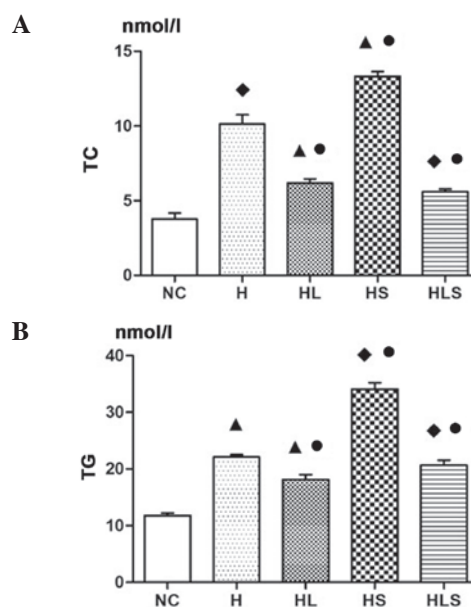


Figure 1. Effects of LBP supplementation and chronic composite psychological stress on (A) TC and (B) TG levels in the mice. The data are expressed as the mean  $\pm$  standard deviation (n=9). ▲ $P < 0.05$  and ♦ $P < 0.01$ , vs. the NC group; ♦ $P < 0.05$ , vs. the H group. LBP, *Lycium barabrum* polysaccharides; TC, total cholesterol; TG, triglycerides; NC, normal control; H, hyperlipidemia group; HS, hyperlipidemia + stress group; HL, hyperlipidemia + LBP group; HLS, hyperlipidemia + stress + LBP group.

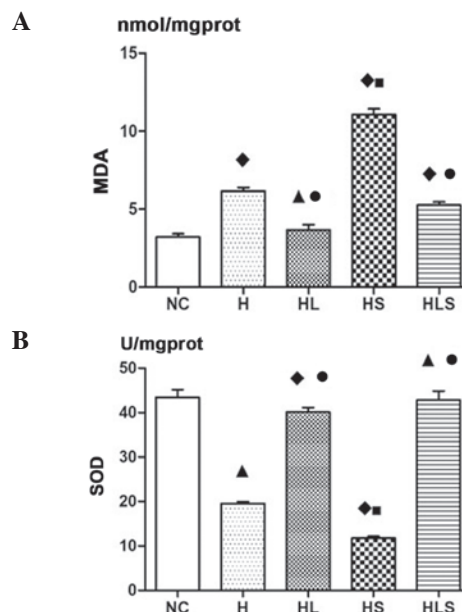


Figure 2. Effects of LBP supplementation and chronic composite psychological stress on hepatic (A) MDA and (B) SOD levels in the mice. The data are expressed as the mean  $\pm$  standard deviation (n=9). ▲ $P < 0.05$  and ♦ $P < 0.01$ , vs. the NC group; ♦ $P < 0.05$  and ♦ $P < 0.01$ , vs. H group. LBP, *Lycium barabrum* polysaccharides; MDA, malondialdehyde; SOD, superoxide dismutase; NC, normal control; H, hyperlipidemia group; HS, hyperlipidemia + stress group; HL, hyperlipidemia + LBP group; HLS, hyperlipidemia + stress + LBP group.

determine whether LBP had any effects on lipid metabolism by detecting changes to blood lipid levels induced by chronic composite psychological stress in hyperlipidemic mice. In all of the hyperlipidemic mice groups TG and TC levels were significantly increased ( $P < 0.05$  and  $P < 0.01$  respectively), as



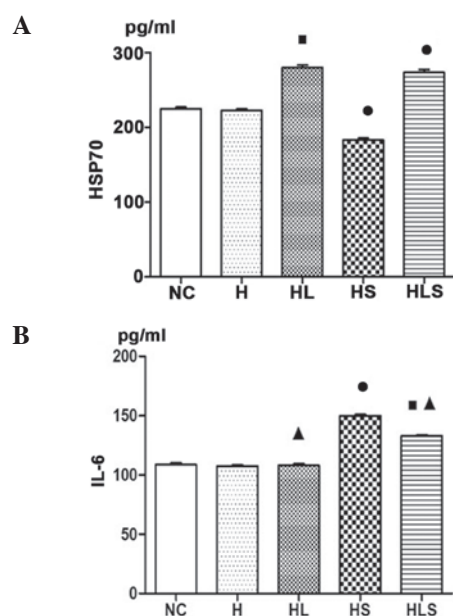


Figure 3. Effects of LBP supplementation and chronic composite psychological stress on the serum expression levels of (A) HSP-70 and (B) IL-6 in the mice. The data are expressed as the mean  $\pm$  standard deviation ( $n=9$ ).  $\blacktriangle P<0.05$ , vs. the HS group;  $\bullet P<0.05$  and  $\blacksquare P<0.01$ , vs. the H group. LBP, *Lycium barbarum* polysaccharides; HSP-70, heat shock protein 70; IL-6, interleukin-6; NC, normal control; H, hyperlipidemia group; HS, hyperlipidemia + stress group; HL, hyperlipidemia + LBP group; HLS, hyperlipidemia + stress + LBP group.

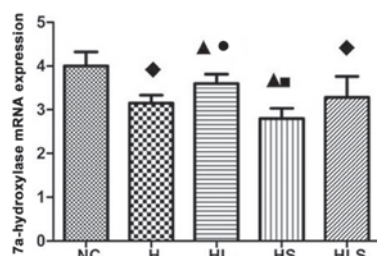


Figure 4. Effects of LBP supplementation and chronic composite psychological stress on the mRNA expression levels of cholesterol 7 $\alpha$ -hydroxylase in the liver of the mice. Data are expressed as the mean  $\pm$  standard deviation ( $n=9$ ).  $\blacktriangle P<0.05$  and  $\bullet P<0.01$ , vs. the NC group;  $\bullet P<0.05$  and  $\blacksquare P<0.01$ , vs. the H group. LBP, *Lycium barbarum* polysaccharides; NC, normal control; H, hyperlipidemia group; HS, hyperlipidemia + stress group; HL, hyperlipidemia + LBP group; HLS, hyperlipidemia + stress + LBP group.

compared with the NC group, whereas the TG and TC levels were higher in the HS group, as compared with the H group ( $P<0.05$ ; Fig. 1A and B). However, TG and TC levels in the HL and HLS groups were significantly lower, as compared with the H group ( $P<0.05$ ). These results indicate that LBP was able to decrease blood lipid levels, in order to attenuate chronic composite psychological stress-induced disordered lipid metabolism.

**Effects of LBP on oxidative stress function following chronic composite psychological stress in hyperlipidemic mice.** MDA is the product of polyunsaturated fatty acid oxidation by ROS in the membrane, and the amount of MDA can reflect the degree of lipid peroxidation and the degree of cellular oxidative stress (14). Measuring MDA levels is one of the most widely used approaches for evaluating oxidative damage to lipids. The

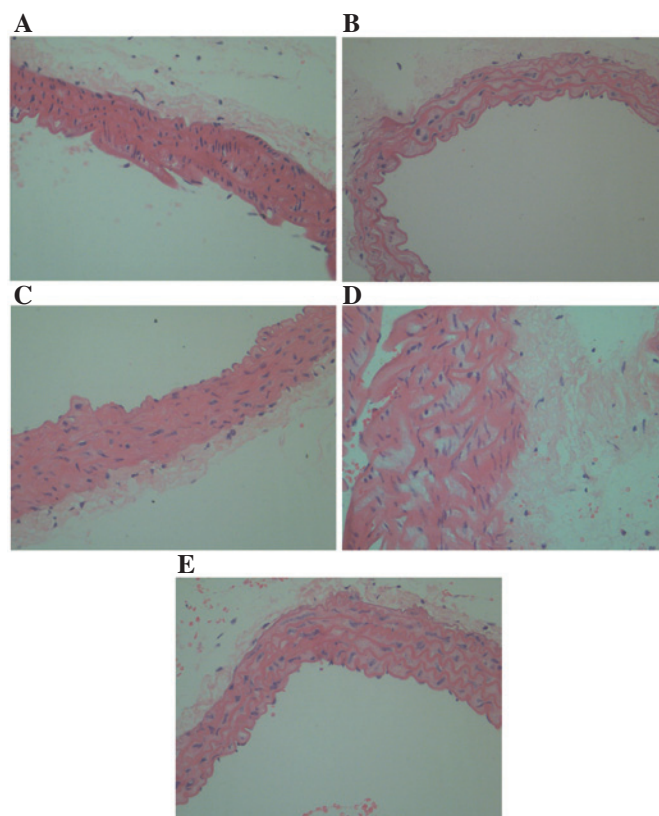


Figure 5. Effects of LBP supplementation and chronic composite psychological stress on morphological changes to the thoracic aorta in the mice, as visualized using hematoxylin and eosin staining (magnification,  $\times 40$ ). Thoracic aorta in the (A) NC group; (B) H group; (C) HL group; (D) HS group; and (E) HLS group by hematoxylin and eosin staining (magnification,  $\times 40$ ).

levels of hepatic MDA were higher in each of the hyperlipidemia groups, as compared with the NC group ( $P<0.05$  and  $P<0.01$ , respectively); whereas the levels of hepatic MDA in the HS group were significantly increased, as compared with the H group ( $P<0.01$ ; Fig. 2A). The levels of hepatic MDA in the HL and HLS groups were lower, as compared with the H group ( $P<0.05$ ). These results indicate that LBP was able to attenuate chronic composite psychological stress-induced lipid peroxidation.

It is well known that SOD can inhibit oxidative stress to protect cells from oxidative damage through the removal of excess ROS. Decreased SOD activity can result in increased levels of oxygen free radicals, therefore SOD is regarded as an indirect marker of the ability of the body to clear oxygen free radicals (15). Therefore the present study evaluated the hepatic levels of SOD. The levels of hepatic SOD in each of the hyperlipidemia groups was significantly lower, as compared with the NC group ( $P<0.05$  and  $P<0.01$ , respectively), whereas the levels of hepatic SOD in the HS group were decreased, as compared with the H group ( $P<0.01$ ; Fig. 2B). The levels of hepatic SOD in the HL and HLS groups were higher, as compared with the H group ( $P<0.05$ ). These results suggest that LBP may be able to increase antioxidant enzyme activities, in order to attenuate chronic composite psychological stress-induced oxidative stress.

**Effects of LBP on biochemical parameters following chronic composite psychological stress in hyperlipidemic mice.** HSP-70

is a group of highly conserved binding proteins induced by numerous stressors, which exhibit a cardioprotective role by reducing oxidative stress and regulating cell functions (16). To determine the expression levels of HSP-70 following chronic composite psychological stress and treatment with LBP, the serum levels of HSP-70 were analyzed by ELISA. An immediate increase in HSP-70 levels were observed in the HL and HLS groups ( $P<0.05$  and  $P<0.01$ , respectively). The serum levels of HSP-70 were significantly lower in the HS group, as compared with the H group ( $P<0.05$ ; Fig. 3A). These results suggest that treatment with LBP was able to increase the expression levels of HSP-70, to attenuate chronic composite psychological stress-induced oxidative stress.

Inflammation serves as the initial pathological step of various cardiovascular diseases, including AS (17). The results of the present study demonstrated that there was a significant increase in the expression levels of IL-6 in the HS group, as expected. In addition, the expression levels of IL-6 were significantly reduced in response to treatment with LBP (Fig. 3B). These results suggest that increased expression levels of IL-6 induced by chronic composite psychological stress, may be reversed by treatment with LBP.

**mRNA expression levels of CYP7A1.** CYP7A1 is a rate-limiting enzyme that converts cholesterol into bile acid, during lipid metabolism in the liver (18). The present study examined the effects of LBP on the mRNA expression levels of CYP7A1 in the livers of mice following chronic composite psychological stress. The mRNA expression levels of CYP7A1 were increased in the HL group compared with those of the H and HS groups ( $P<0.05$ ; Fig. 4). However, the expression levels of CYP7A1 were significantly attenuated in the hyperlipidemic mice, following chronic composite psychological stress ( $P<0.01$ ). The expression levels of CYP7A1 were increased in the HLS group, as compared with the H group, however this finding was not statistically significant ( $P>0.05$ ). These results indicate that treatment with LBP was able to significantly reverse the inhibition of CYP7A1 mRNA expression in mice following chronic composite psychological stress.

**Morphological observation of the thoracic aorta.** In the thoracic aorta of the NC group, aligned cells and clear demarcations were observed between the intima, medial and outer membranes (Fig. 5). The tubular wall of the thoracic aorta was thicker and the arrangement of cells was slightly disordered in the H group. As well as complete and flat intima, the tubular wall of the thoracic aorta was slightly thicker and the muscular layer of the medial membrane was neatly arranged in the HL group. The HLS group exhibited endothelial cell loss and increased thickness of the intima. In the HS group there were typical atherosclerotic changes to the tubular wall of the thoracic aorta, as well as disordered cell arrangement, endothelial cell loss, reduction in smooth muscle cells and hyperplasia of fibrous tissue.

## Discussion

The present study investigated the effects of LBP on oxidative stress following chronic composite psychological stress intervention in hyperlipidemic mice. A major finding of the

study was the increased levels of TC and TG in the hyperlipidemic mice subjected to chronic composite psychological stress. However, supplementation with LBP for 30 days markedly reduced blood lipid levels in the HL and HLS groups, as compared with that in the H group. A previous study demonstrated that the hypothalamic pituitary adrenal axis, the sympathetic adrenal medullary axis and the renin-angiotensin-aldosterone system were all capable of releasing large quantities of stress hormones following chronic composite psychological stress, including catecholamine, adrenal cortical hormone, glucagon, thyroxine and rennin (19). Catecholamine and adrenal cortical hormone have previously been shown to decrease the scavenging ability of TG on vascular endothelial cells by inhibiting lipoprotein lipase activity. However, large quantities of catecholamine and glucagon have been demonstrated to promote fat decomposition into TG, phospholipid and cholesterol, in order to accelerate the release of free fatty acids, eventually leading to elevated blood lipids (20). The present study demonstrated that CYP7A1 mRNA expression levels were lower in the HS group, as compared with the H group. These results indicate that chronic composite psychological stress was able to stimulate the body to release stress hormones, in order to accelerate the decomposition of fat tissue, and to decrease decomposition of cholesterol in the liver by inhibiting CYP7A1 expression. These effects led to increased blood lipid levels in the HS group. Furthermore, treatment with LBP was able to decrease the chronic composite psychological stress-induced blood lipid levels, to attenuate disordered lipid metabolism, which may be associated with increased expression levels of CYP7A1 in the liver.

Generally, disordered lipid metabolism impairs oxidative stress function and results in lipid peroxidation (21). The present study demonstrated that chronic composite psychological stress resulted in a significant increase in oxidative stress, which decreased the levels of the antioxidant enzyme SOD, and increased the levels of the lipid hydroperoxide MDA in the HS group. These results suggest that chronic composite psychological stress-induced disordered lipid metabolism may induce lipid peroxidation, which damages the vascular endothelial cells and aggravates lipid deposition, initiating the process of AS (22). A previous study showed that LBP is effective in avoiding oxidative stress and can clear excess free radicals and decrease the levels of lipid peroxidation (23). Combined with the results of the present study, LBP may be considered a useful protective agent in hyperlipidemic mice subjected to chronic composite psychological stress. However, whether LBP is helpful for patients with AS requires further confirmation.

A previous epidemiological survey suggested that increased levels of serum proinflammatory cytokines were associated with acute psychological stress, depression, anxiety, hostility and long-term low socioeconomic status (24). The present study demonstrated that the serum levels of IL-6 were significantly increased in the HS group, and the aortic wall also exhibited typical atherosclerotic morphological changes. These results indicate that chronic composite psychological stress may induce vascular inflammation and promote the occurrence and development of AS, through increasing serum levels of IL-6. There are numerous possible underlying mechanisms regarding the effects of chronic composite psychological stress on AS, one hypothesis was that chronic composite psychological

stress induced lipid peroxidation through the production of excess oxygen free radicals, which resulted in the disruption of the balance between free radicals and oxygen inhibitors, therefore increasing the formation of oxidized low density lipoproteins, which are thought to be key mediators of the AS inflammatory reaction (25). Another possible mechanism is the excessive release of stress hormones by the hypothalamic pituitary adrenal axis, the sympathetic adrenal medullary axis and the renin-angiotensin-aldosterone system, including catecholamine, adrenal cortical hormone, glucagon, thyroxine and rennin. These hormones are able to elevate blood pressure and heart rate, and cause intravascular hemodynamics, increasing the expression of Toll-like receptors (26), and activating nuclear transcription factor, thus priming the inflammatory response and increasing vascular endothelial injury (27). The present study hypothesized that chronic composite psychological stress promoted the occurrence and development of AS by initiating disordered lipid metabolism, promoting lipid peroxidation and priming vascular inflammation. Therefore, delaying the development of AS may be achieved by maintaining the balance of lipid metabolism, preventing lipid peroxidation and protecting endothelial function.

The participation of HSPs in the immune response following brain injury may be considered an attempt to correct the inflammatory condition (28), which is essential for modulating cellular function and maintaining protein homeostasis. In the HL and HLS groups, the HSP-70 levels were significantly increased, as compared with those in the H group. These results suggest that LBP may reduce oxidative stress and protect vascular endothelial cells through increasing the expression levels of endogenous protective substances, such as HSP-70.

In conclusion, the present study clearly indicated that LBP was able to reduce blood lipid levels and enhance antioxidative effects in hyperlipidemic mice following chronic composite psychological stress, which may be due to a reduction in lipid peroxidation and an enhancement of lipid metabolism. However, the underlying mechanisms of regulation require further investigation in order to use this strategy to delay the development of AS.

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