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Original Article

Heat shock protein expression affects high-density lipoprotein function in atherosclerosis

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Abstract: High-density lipoprotein (HDL) and heat shock protein (HSP) play important role in atherosclerosis (AS) lesion. This study aimed to investigate the relationship between HDL function and HSP65 in AS by observing different doses of HSP65 impact on macrophage cholesterol efflux rate in apolipoprotein E gene deleted mice and HDL anti-inflammatory, antioxidant function. Healthy male ApoE$^{-/-}$ mice at 8 weeks were fed with high-fat diet. On the 16th week, blood lipid, serum IFN-γ, IL-10, MPO, PON1 levels were tested. HSP65 antibody titer and peritoneal macrophages ^{3}H-TC switching-out rate were determined. Liver tissue ABCA1, ABCG1, SR-BI, and LXR-α protein expression were detected by Western blot. TG, TC, and LDL-C level elevated, while HDL-C decreased significantly in group B, C, D, and E compared with group A ($P < 0.05$). Group E showed lower HDL-C level than group B ($P < 0.05$). HSP65 antibody titer, IFN-γ, and IL-10 level in group B were similar to group A ($P < 0.05$). HSP65 antibody titer and IFN-γ level increased, whereas IL-10 reduced in group C, D, and E compared with group B with dose-dependent ($P < 0.05$). Macrophages ^{3}H-TC switching-out rate, PON1 activity, and liver tissue ABCA1, ABCG1, SR-BI, and LXR-α protein level declined obviously, while MPO activity increased significantly in group B, C, D, E compared with group A ($P < 0.05$). HSP65 induced inflammatory immunoreaction, damaged HDL function, and declined macrophage cholesterol efflux rate through downregulating liver tissue ABCA1, ABCG1, SR-BI, and LXR-α protein with dose-dependent.

Keywords: Atherosclerosis, HDL, HSP, LXR-α, macrophage cholesterol efflux rate

Introduction

Atherosclerosis (AS) is an important reason to cause multiple cardiac-cerebral vascular diseases. It is featured as dyslipidemia, vascular endothelial cells injury, and vascular chronic inflammatory immune response [1, 2]. Present study showed that AS was promoted by oxidative modification of monocyte-derived-macrophages, lipoprotein, T lymphocytes, and normal cell components in the arterial wall [3, 4]. High-density lipoprotein (HDL) has various functions including anti-oxidation, anti-inflammation, promoting reverse cholesterol transport (RCT), antithrombus, and improving endothelial function. AS was negatively correlated with HDL-C, though HDL-C cannot accurately reflect HDL function [5, 6]. Under acute and chronic inflammation reaction condition, HDL composition and function changes lead to its lack of normal function. Thus, exploring HDL function changes has important significance for AS and CHD risk assessment [7, 8]. The main physiological function of heat shock protein (HSP) is featured as protein structure catalyst that has critical role in cell proliferation, cell cycle regulation, and apoptosis. HSP can activate autoimmune reaction to promote cytokines secretion, because of HSP cross reaction between different species and highly conserved sequence. The pathogenesis of B and T cell response indicated that HSP was related to various inflammatory response and autoimmune reaction. HSP65 has strong immunogenicity that overexpressed in infection. It can promote AS pathological process through the inflammatory reaction. Animal experiments showed that AS plaque area increased significantly in mice with HSP65 immunity, and proinflammatory factor expression within the plaque elevated obviously [9, 10]. Inflammatory stimulation can promote HDL function change, while whether HSP65 can trigger HDL function change is still unclear. Th-

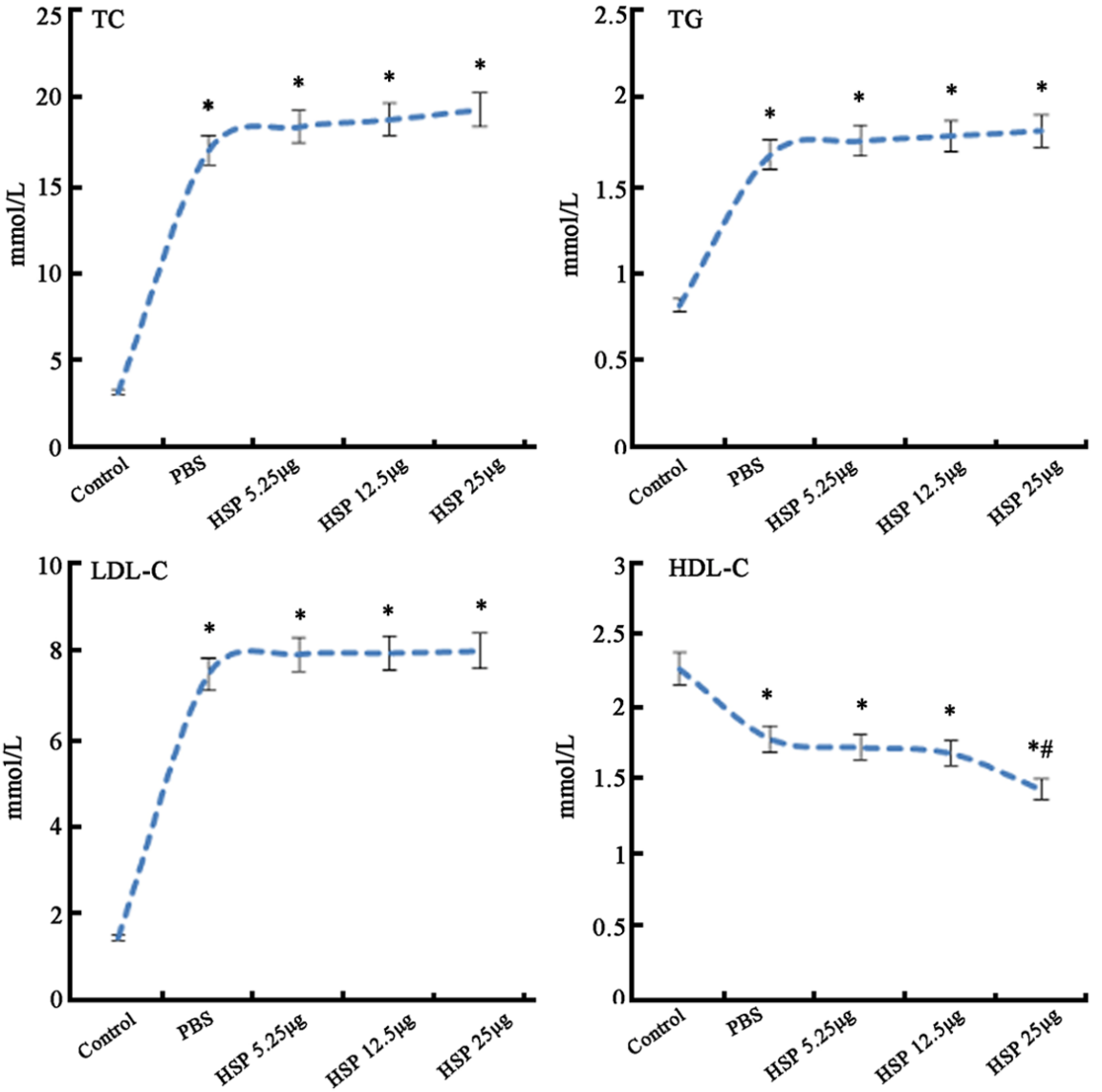

Figure 1. Blood lipid level comparison. Group A, control; group B, PBS; group C, HSP 6.25 μg; group D, HSP 12.5 μg; group E, HSP 25 μg. *$P < 0.05$, compared with group A; #$P < 0.05$, compared with group B.

is study intended to discuss the relationship between HDL function changes and HSP65 in AS through injecting different doses of HSP65 to ApoE$^{-/-}$ mice to observe its impact on macrophage efflux rate and HDL function.

Materials and methods

Experimental animal and grouping

Ten healthy male C57BL/6J mice at 8 weeks old and weighted 18~22 g were fed normally as the control group (A). Forty healthy male ApoE$^{-/-}$ mice provided by the Chinese academy of medical sciences, animal experiment center (license SYXK-2013-0025). The mice were maintained in SPF grade laboratory animal center. ApoE$^{-/-}$ mice were randomly divided into phosphate buffer solution (PBS) group (B), 6.25, 12.5, and 25 μg HSP65 group (C, D, E). The mice in group A were fed with common diet for 16 weeks, while the mice in group B, C, D, and E received high-fat diet for 16 weeks. They received PBS or HSP65 back subcutaneous injection at the 3rd or 16th week at 20 ml/kg.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics

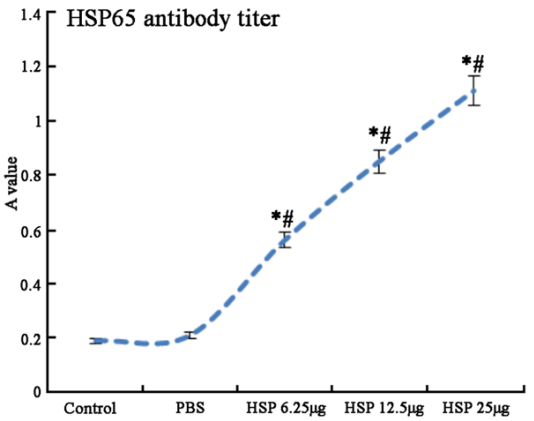

Figure 2. Serum HSP65 antibody titer comparison. $^{*}P < 0.05$, compared with control; $^{\#}P < 0.05$, compared with PBS.

Committee of Central Hospital of Chongqing Three Gorges.

Test drugs and reagents

HSP65 was from StressMarq. RAW264.7 macrophages were provided by ATCC. ^{3}H-TC was purchased from Perkin Elmer. IFN-γ and IL-10 ELISA kits, MPO, PON1, and total cholesterol kits were bought from Nanjing Jiancheng Bioengineering Institute. ABCA1 primary antibody was from Novus. ABCG1, SR-BI, and LXR-α primary antibodies were from Abcam.

Specimen collection

On the 16th week, after fasted for 12 h, the blood was extracted from heart to isolate serum. Peritoneal macrophage and liver tissue were stored in liquid nitrogen. Aorta was separated under the microscope and fixed with 10% formaldehyde.

Serum lipid, IFN-γ, IL-10, MPO, PON1 level detection

Blood lipid was tested by automatic biochemistry analyzer: total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-C), HDL-C. IFN-γ and IL-10 were detected by ELISA. MPO and PON1 levels were tested according to the manual.

Serum HSP65 antibody titer determination

According to previous report [11], antibody (protein content 1 μg/ml) was diluted by PBS and added in polystyrene board at 4°C overnight. Then the plate was blocked by BSA after washing. After adding serum and incubated at 37°C for 1 h, the plate was then added with horseradish peroxidase at 37°C for 1 h. Then the plate was treated with tetramethyl benzidine substrate solution at 37°C for 30 min and terminated by sulfuric acid. At last, the plate was read on microplate reader at 450 nm.

Macrophage ^{3}H-TC switching-out rate determination

RAW264.7 macrophages were seeded in 24-well plate at 2 × 10^{5} cells/ml and cultured with DMEM containing 0.2% BSA, 1 μCi/ml ^{3}H-TC and 30 μg/ml oxidized LDL [12, 13]. After 24 h incubation and washed by PBS for 2 times, the macrophages were added with DMEM containing 2.8% mice serum DMEM for 6 h. After treated by NaOH at room temperature for half an hour, the cell lysis was collected and detected using liquid flash counter. ^{3}H-TC switching-out rate = ^{3}H-TC radiation quantity in cytokine/(^{3}H-TC radiation quantity in cytokine + intracellular).

Western blot

Mice liver tissue was cracked by lysis and protein content was determined by BCA kit. The protein was separated by SDS-PAGE and transferred to PVDF membrane. After blocked for 1 h, the membrane was incubated in primary antibody (ABCG1 1:1000, ABCA1 1:500, SR-BI 1:500, LXR-α 1:500) at 4°C overnight. And then the membrane was incubated in secondary antibody (1:15000) for 1 h and developed by luminescence reagent. The image was analyzed by Quantity One software.

Aortic plaques determination

Aorta was extracted and fixed. After dehydration, hyalinization, embedding, sectioning, and HE staining, the slice was observed under the microscope and analyzed by Image-plus 6.0 software. The area ratio of plaque and blood vessel was calculated.

Statistical analysis

SPSS 19.0 was applied to perform normality test. Measurement data in normal distribution was presented as mean ± standard deviation ($\bar{X} \pm S$). One-way ANOVA and LSD test was used

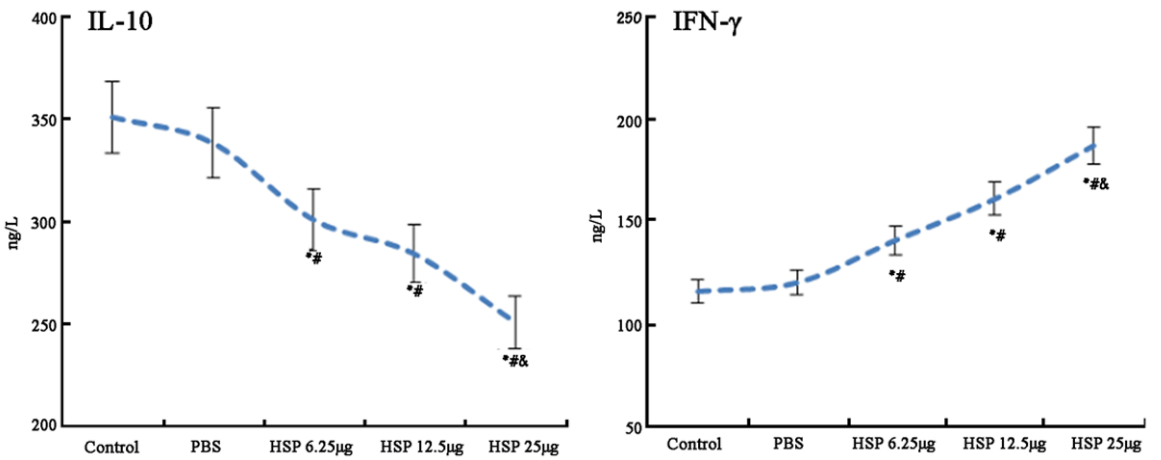

Figure 3. Serum IFN-γ and IL-10 comparison. *$P < 0.05$, compared with control; #$P < 0.05$, compared with PBS.

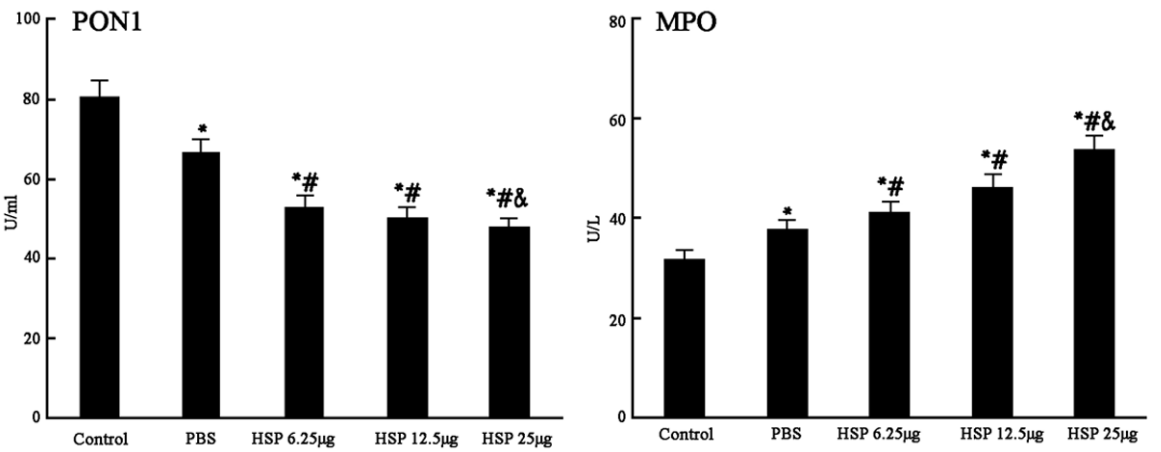

Figure 4. Serum MPO and PON1 comparison. *$P < 0.05$, compared with control; #$P < 0.05$, compared with PBS; &$P < 0.05$, compared with HSP 6.25 μg.

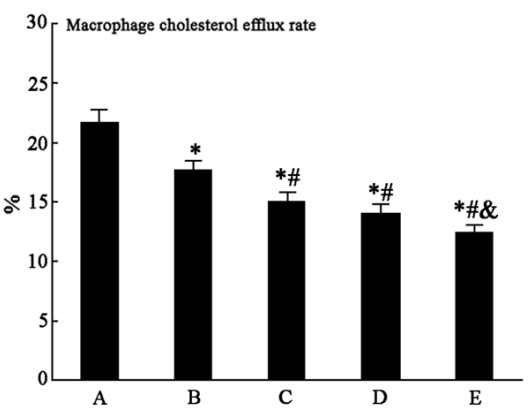

Figure 5. Macrophage cholesterol efflux rate comparison.

for mean value comparison. $P < 0.05$ was considered as significant difference.

Results

Blood lipid level comparison

Serum TG, TC, LDL-C levels in group B, C, D, E increased, while HDL-C decreased significantly compared with group A ($P < 0.05$). No obvious difference was observed in serum TG, TC, LDL-C among group B, C, D, and E ($P > 0.05$). Group E showed markedly lower HDL-C level than that in group B ($P < 0.05$) (**Figure 1**).

Serum HSP65 antibody titer comparison

Group B showed no statistical difference of HSP65 antibody titer with group A ($P > 0.05$). HSP65 antibody titer increased significantly in group C, D, and E compared with group B with dose-dependent ($P < 0.05$) (**Figure 2**).

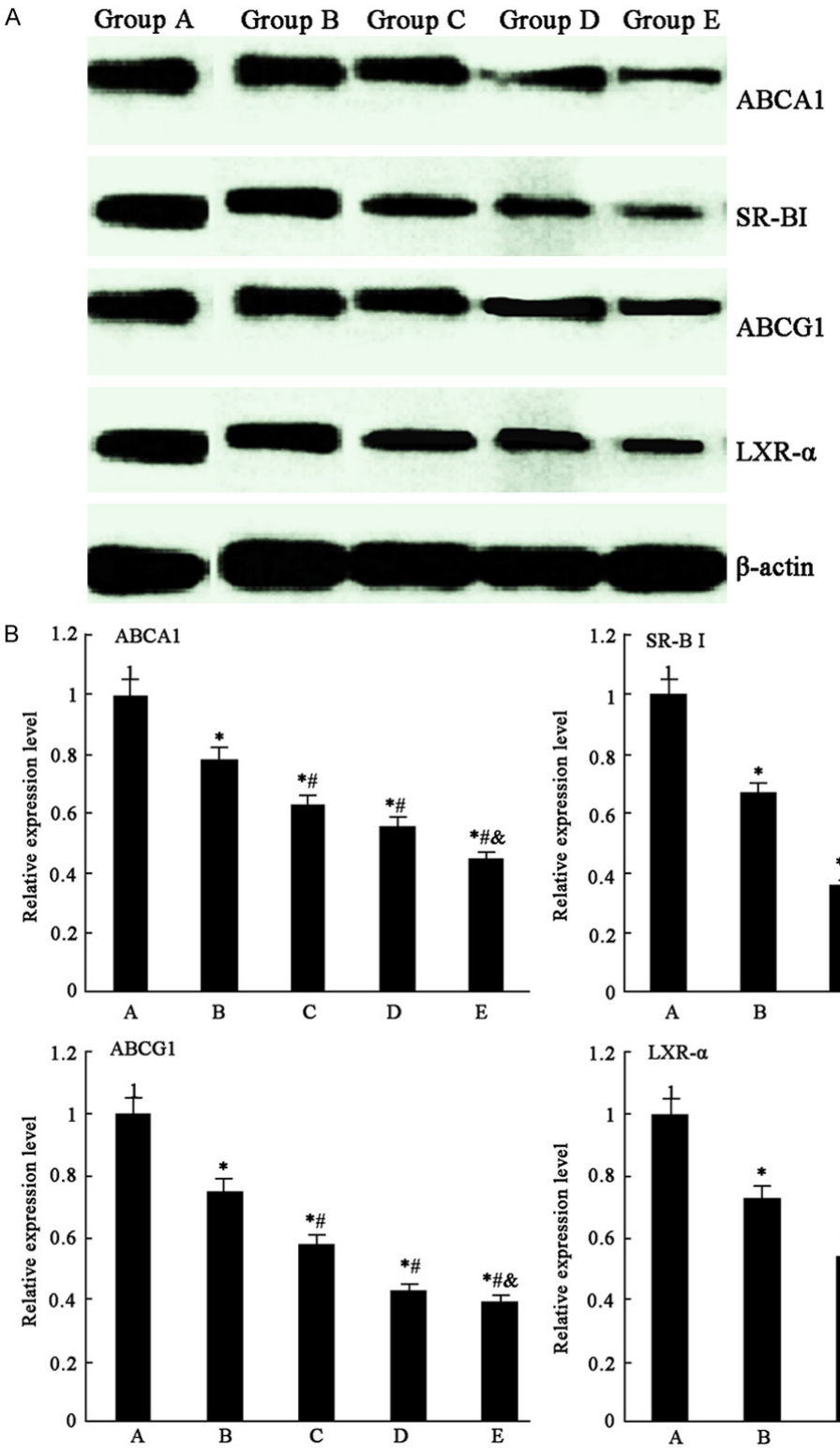

Figure 6. A. ABCA1, ABCG1, SR-BI, and LXR-α protein expression in liver tissue. B. ABCA1, ABCG1, SR-BI, and LXR-α protein expression level in liver tissue.

Serum IFN-γ and IL-10 comparison

AS progress is associated with Th1/Th2 imbalance. As an inflammation suppressor, IL-10 is mainly secreted by Th2. It plays an anti-AS effect through downregulating cell adhesion molecule expression, and inhibiting proinflammatory factor effect and immune response.

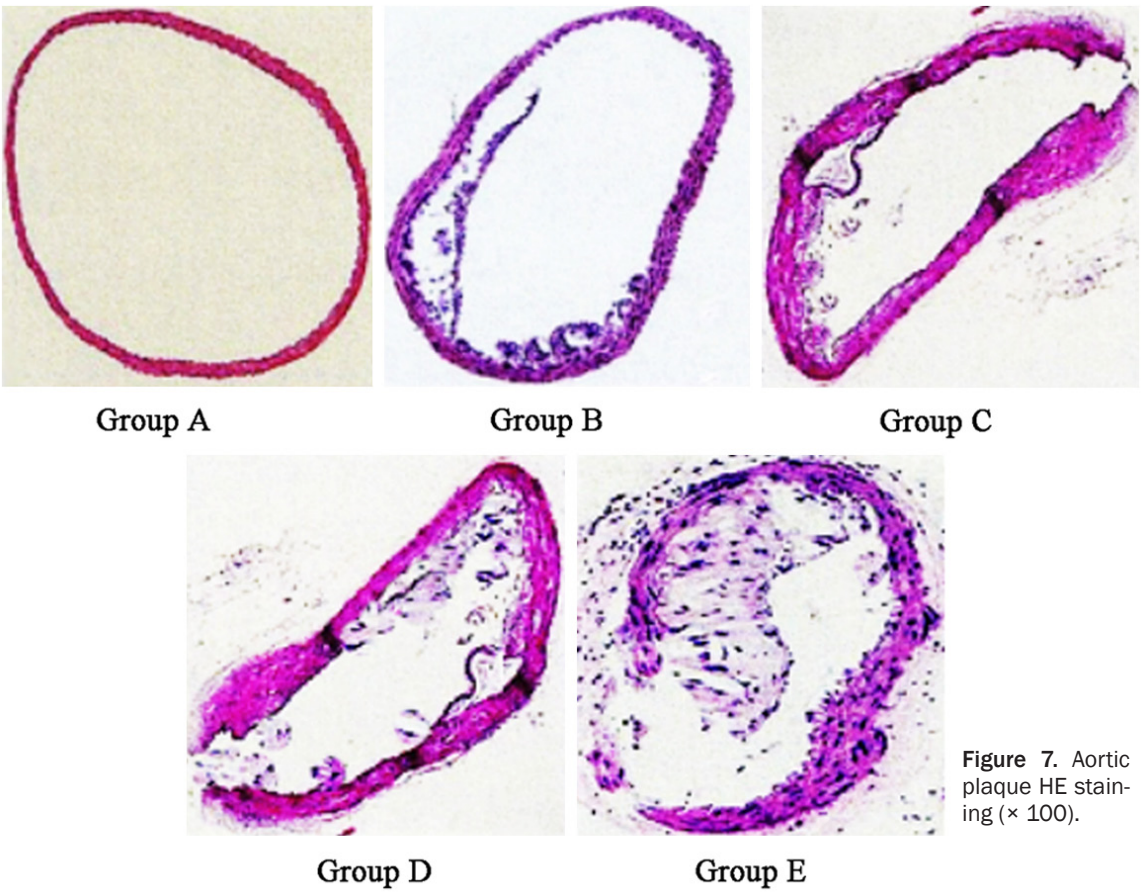

Figure 7. Aortic plaque HE staining (× 100).

IFN-γ is a proinflammatory factor mainly secreted by Th1. It can promote AS by collaboratively inducing inflammatory factor expression. Compared with group A, IFN-γ and IL-10 showed no significant difference in group B ($P > 0.05$), while IL-10 declined and IFN-γ elevated in group C, D, and E group with dose dependent (**Figure 3**).

Serum MPO and PON1 comparison

PON1 level reduced while MPO level rise in group B compared with group A ($P < 0.05$). Following dose increasing, PON1 declined and MPO elevated in group C, D, and E with dose-dependent (**Figure 4**).

Macrophage cholesterol efflux rate comparison

Macrophage cholesterol efflux rate decreased obviously in group B compared with group A ($P < 0.05$). Group C, D, and E presented significant macrophage cholesterol efflux rate reduction with dose-dependent (**Figure 5**).

ABCA1, ABCG1, SR-BI, and LXR-α protein expression in liver tissue

ABCA1, ABCG1, SR-BI, and LXR-α protein expression level decreased markedly in group B compared with group A ($P < 0.05$), whereas they reduced obviously in group C, D, and E (**Figure 6**).

Aortic plaque HE staining

HE staining revealed that HE staining showed smooth intima and integrate endothelial cells in group A. Intima thickening and plaque area increasing could be observed in group B, C, D, and E (**Figure 7**).

Discussion

The risk of cardiovascular disease reduces 2% following HDL-C level elevates 1 mg/dL. Several CHD patients show HDL-C reduction [14, 15]. Clinical trial showed that the incidence of cardiovascular events cannot be reduced by increasing HDL-C level. Cholesterol transport pro-

tein inhibitor can increase the serum HDL-C level, but could not prevent AS progress, as HDL-C level cannot fully reflect HDL function [16, 17]. Reverse cholesterol transport is an important way of HDL anti-AS, including intracellular cholesterol efflux-cholesterol esterification-in vivo excretion. Too much cholesterol accumulation can cause macrophage bubblization and promote AS progress. It was found that the risk of CHD was negatively correlated with macrophage cholesterol efflux rate, and independent to HDL-C level that can be treated to evaluate HDL function [18, 19]. HDL promoting reverse cholesterol transport and anti-inflammatory antioxidation play an important role in AS process. HDL can play its anti-inflammation role by inhibiting monocyte adhesion and decreasing endothelial cell adhesion molecule expression. HDL exerts antioxidant effect through inhibiting LDL oxidation of phospholipids and inactivated generated phospholipids, while PON1 and MPO are the most important enzymes in the process. PON1 has various functions including preventing lipid peroxide generation, inhibiting LDL oxidation, hydrolysis of cholesterol ester hydroperoxide type phospholipid, and eliminating LPO in LDL. MPO may cause HDL losing anti-AS effect by oxidizing apolipoprotein A-I in tissue and blood circulation [19, 20]. Detecting MPO and PON1 activity can reflect the antioxidant function of HDL. Our study revealed that HSP65 subcutaneous immunization on ApoE$^{-/-}$ mice can cause serum IL-10 decline, IFN-γ elevation, decrease PON1 activity, and elevate MPO activity with dose-dependent, suggesting that HDL function change is associated with inflammation. HSP65 subcutaneous immunization on ApoE$^{-/-}$ mice may induce inflammation and promote HDL function changes, further leading to AS progress. In this study, HSP65 subcutaneous injection reduced macrophage efflux rate with dose-dependent, indicating that HSP65 impaired reverse cholesterol transport mediated by HDL and promoted AS except inducing inflammation.

HSP can be divided into different subgroups in accordance with amino acid sequence homology and relative molecular weight, such as HSP70, HSP60, and HSP90. Multiple studies have shown that oxidative stress and oxidized low density lipoprotein can induce HSP overexpression in macrophages and endothelial cells mainly mediated by HSP-1, leading to macrophages and T lymphocyte activation, inducing immune response to promote AS. HSP65 antibody titer increased following the AS degree elevation. HSP65 can induce inflammation and aggravate inflammation through NF-κB signaling pathway by binding with TLR4/CD14 [19, 20]. Multiple factors regulate the process of macrophage cholesterol efflux. Current research showed that the active efflux way containing ABCG1, ABCA1, and SRBI mediated extracellular receptor combination, promoting macrophage cholesterol efflux [20, 21]. Liver is the key position of reverse cholesterol transport process. The main effect of ABCG1 is to mediate cholesterol transporting to the mature HDL. ABCA1 can promote intracellular pre-HDL formation by combining phospholipids to free cholesterol and apolipoprotein A I, which is the rate-limiting step in the process. The real HDL receptor is SRB I, while the later mediates intracellular cholesterol efflux and liver tissue uptake HDL-C, promoting bidirectional cholesterol efflux between HDL and cells [22-24]. LXR-α is one of the major nuclear receptors for regulating cholesterol metabolism that can promote intracellular free cholesterol efflux through regulating ABCG1 and ABCA1 expression. Inflammatory immune response can reduce cholesterol transport protein expression level, and inflammatory factors IL-1, IFN-γ, and TNF-α can reduce ABCG1 expression level [23, 24]. This study revealed that HSP65 subcutaneous injection on ApoE$^{-/-}$ mice gradually declined macrophage efflux rate with dose-dependent, decreased ABCA1, ABCG1, SR-BI, and LXR-α protein expression in liver tissue, suppressed serum IL-10 level, and elevated IFN-γ level, suggesting that HSP65 can cause macrophage efflux rate reduction and ABCA1, ABCG1, SR-BI, and LXR-α protein downregulation in liver tissue. In conclusion, HSP65 can reduce macrophage cholesterol efflux rate with dose dependent by down-regulating ABCA1, ABCG1, SR-BI, and LXR-α protein expression, inducing inflammation, and damaging HDL function.

Disclosure of conflict of interest

None.

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