Experimental study of the anti-atherosclerotic effect of demethylzeylasteral

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Abstract. This study aimed to confirm that atherosclerosis (AS) is a systemic immune-mediated chronic inflammatory disease and to investigate the anti-atherosclerotic effect of demethylzeylasteral by testing the immunocompetent cells and inflammatory mediators in the blood and atherosclerotic plaques of the rabbit model of AS. For this purpose, 60 male New Zealand white rabbits were given 150 g high-fat diet (1% cholesterol, 5% lard, and 15% egg yolk powder) daily for a total of 90 days. On day 61, the rabbits were randomly divided into the saline group (n=15), the rosuvastatin group (n=15), the low-dose demethylzeylasteral group (n=15), and the high-dose demethylzeylasteral group (n=15). The $CD3^+$ T lymphocytes and the subsets $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$, as well as the soluble interleukin-2 receptor (sIL-2R) were measured before and after the treatment. The contents of immunoglobulins IgG, IgA and IgM and the levels of complements C3 and C4 were also monitored. In addition, the level of anti-oxidized low-density lipoprotein (ox-LDL) antibody, the inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-6 and metalloproteinase-9 (MMP-9), the blood lipids triglyceride (TG), total cholesterol (TC), LDL cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured, and the severity of plaque lesions was also evaluated. Our results showed that the saline group, the rosuvastatin group and the low-dose demethylzeylasteral group had significantly lower activated T lymphocyte parameters $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ ($P<0.05$), and significantly higher levels of sIL-2R, immunoglobulins IgG, IgA and IgM, complements C3 and C4, anti-ox-LDL antibody, TNF-α, IL-6 and MMP-9 ($P<0.01$) when compared with the high-dose demethylzeylasteral group. Moreover, TG, TC, LDL-C contents were found significantly lower and their HDL-C contents were significantly higher in high-dose demethylzeylasteral group ($P<0.01$) as compared to the other three groups. Furthermore, Sudan staining and haematoxylin and eosin staining of the thoracic aorta showed that, after 30-day treatment, the high-dose demethylzeylasteral group had the smoothest intima and the lightest plaque lesions among the four groups. Based on these results, we concluded that AS is a systemic immune-mediated chronic inflammatory disease and the relatively high dose of demethylzeylasteral used in the treatment of atherosclerotic rabbits could significantly alleviate AS. This implies that demethylzeylasteral may be considered as a suitable drug for anti-immunization therapy.

Introduction

Atherosclerosis (AS), a chronic progressive disease characterized by fiber and lipid depositions on the artery wall (1), is a major cause of myocardial infarction and stroke. The pathogenesis of AS has not been fully understood yet. Recent studies have shown that immunity and inflammatory responses might be involved in the whole process of AS, and it was even suggested that AS was a kind of autoimmune disease (2-5). Macrophages, T lymphocytes and many other immune cells have been detected in the AS lesions. In addition, the incidence of AS was significantly higher in patients with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, and glucocorticoid treatment could alleviate AS (6). The above evidence suggests the existence of similarities between AS and autoimmune diseases.

Coronary heart disease and stroke caused by AS are the main reasons of death in humans. The existing anti-AS drugs primarily reduce low-density lipoprotein cholesterol (LDL-C) (e.g., statins and ezetimibe), and prevent platelet aggregations. However, the effects of these anti-AS drugs are still unsatisfactory. Studies showed that anti-inflammatory and anti-immune drugs such as cyclosporine and methotrexate could reduce or delay the progression of AS (7-10), but whether anti-inflammatory and anti-immune Chinese medicine *Tripterygium* would reduce or delay AS has not been reported. Therefore, the main

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focus of this study was to explore the anti-atherosclerotic effect of demethylzeylasteral.

Materials and methods

Animals. For this study, 60 male New Zealand white rabbits (weight, 2.0-2.5 kg) were housed in the SPF level animal facility with one rabbit per cage. Feed and water was given to the animals, and litter and cages were sterilized before use. The indoor temperature was kept at 20-22˚C, and the animals had each day 12-h light and 12-h dark.

Animal feed and demethylzeylasteral intervention. The rabbits were given 150 g high-fat diet (1% cholesterol, 5% lard and 15% egg yolk powder) daily for 90 days. On day 61, the animals were randomly divided into the saline group, the rosuvastatin group, the low-dose demethylzeylasteral group, and the high-dose demethylzeylasteral group, each consisting of 15 rabbits. The rabbits from each group received saline, 0.5 mg/kg/day rosuvastatin (National Medicine Permit no. J20090092), 10 mg/kg/day demethylzeylasteral (provided by the Department of Pharmacy, Zhongshan Hospital of Fudan University, Shanghai, China) and 40 mg/kg/day demethylzeylasteral, respectively by intragastrical administration. The treatment was continued for 30 days.

Collection of blood samples. Venous blood samples were collected before the high-fat feeding, and before and after the interventions with saline, rosuvastatin and demethylzeylasteral. Ethics approval for animal experiments was from the Animal Ethics Committee of Shanghai University of Medicine and Health Sciences.

Collection of pathology specimen. On day 91, the rabbits were sacrified by air injection after they were given anesthesia by intraperitoneal injection of ketamine (35 mg/kg) and diazepam (5 mg/kg). Then the abdominal cavities of the animals of each group were opened and the epicardial adipose tissues were peeled from the aortic root to the level of the iliac artery. After that, the aortas were stored in 4% paraformaldehyde (PBS, pH 7.4). Then, a 0.5-cm fragment starting from the descending aorta was separated and sliced into 4 μm pathology sections for immunohistochemical [hematoxylin and eosin (H&E)] staining. The rest of the aorta was opened longitudinally and stained with Sudan III.

Measurements of cellular immune indexes. The $CD3^+$ T lymphocytes and the subsets $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ were measured by direct immunofluorescence labeling before and after the treatment. An assay (ELISA) was used to measure the soluble interleukin-2 receptor (sIL-2R) with the sIL-2R kit (Meixuan Biotechnology Co., Ltd., Shanghai, China) by following the manufacturer's operating manual.

Determination of humoral immune indexes. The rate nephelometry was used to determine the serum level of IgG, IgA, IgM, C3 and C4.

Measurement of autoimmune index. The anti-oxidized LDL (ox-LDL) antibody was measured by using ELISA with the anti-rabbit ox-LDL antibody ELISA kit (Nanjing SenBeijia Biotechnology Co., Ltd., Nanjing, China) following the instruction of manufacturer's manual.

Determination of inflammatory cytokines. To measure the serum tumor necrosis factor-α (TNF-α), IL-6, metalloproteinase-9 (MMP-9) contents, radioimmunoassay was used. The relevant kits were purchased from SunBio Biomedical Technology (Beijing, China). The operations were conducted according to the instructions given on the manufacturers' manual.

Determination of blood lipid levels. The contents of blood triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), LDL-C and total cholesterol (TC) were measured on an automatic biochemical analyzer (Shengshida Technology, Wuhan, China) following the instructions' manual of the relevant kits.

Analysis of pathology images. Sudan staining and H&E staining were used to stain the rabbit thoracic aortas and the degrees of plaque lesions for each group were evaluated.

Statistical analysis. To perform the statistical analysis, the SPSS 20.0 (IBM SPSS, Armonk, NY, USA) was used. Countable data were converted into rates and then compared by using the Chi-square test. The measurement data were expressed as the mean ± standard deviation. Furthermore, pairwise comparisons were performed by using the t-test, and F test was used to make comparison among groups. Statistical significance was set at $P<0.05$.

Results

Comparison of cellular immune indexes. The results presented in Table I shows that the high-dose demethylzeylasteral group had significantly higher activated T lymphocytes parameters, that is, $CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ ($P<0.05$), but significantly lower sIL-2R level ($P<0.01$) as compared to other three groups.

Comparison of humoral immune indexes. The results presented in Table II demonstrate that the saline group, the rosuvastatin group, and the low-dose demethylzeylasteral group showed significantly higher levels of immunoglobulins IgG, IgA and IgM, as well as the levels of complements C3 and C4 than the high-dose demethylzeylasteral group ($P<0.05$).

Comparison of autoimmune index. The level of anti-ox-LDL antibody was found significantly higher in the saline group, the rosuvastatin group, and the low-dose demethylzeylasteral group (Fig. 1) than in the high-dose demethylzeylasteral group ($P<0.05$).

Comparison of inflammatory cytokines. The results presented in Table III illustrates that the saline group, the rosuvastatin group, and the low-dose demethylzeylasteral group had significantly higher levels of TNF-α, IL-6 and MMP-9 than the high-dose demethylzeylasteral group ($P<0.01$).

Table I. Comparison of cellular immune indexes between groups.

Group	$CD3^+$, %	$CD4^+$, %	$CD8^+$, %	$CD4^+/CD8^+$	sIL-2R, ng/l
Saline	41.54±5.32	24.67±4.85	14.27±3.18	1.46±0.52	862.57±171.28
Rosuvastatin	47.73±7.16	28.12±5.04	17.05±4.32	1.61±0.44	794.23±153.71
Low-dose demethylzeylasteral	51.35±7.42	32.51±5.37	19.63±4.65	1.75±0.74	726.75±136.33
High-dose demethylzeylasteral	60.05±8.54	36.85±5.83	23.04±5.12	1.93±0.85	676.72±125.13
F	19.23	17.73	14.54	15.32	10.12
P-value	<0.05	<0.05	<0.05	<0.05	<0.01

sIL-2R, soluble interleukin-2 receptor.

Table II. Comparison of humoral immune indexes between groups.

Group	IgG, g/l	IgA, g/l	IgM, g/l	C3, g/l	C4, g/l
Saline	17.14±4.27	5.23±1.16	3.46±1.25	2.54±0.92	0.63±0.18
Rosuvastatin	15.73±4.06	4.85±0.77	3.07±1.03	2.35±0.74	0.52±0.13
Low-dose demethylzeylasteral	12.46±3.85	3.46±0.63	2.37±0.86	1.91±0.54	0.37±0.11
High-dose demethylzeylasteral	9.66±3.17	2.28±0.42	2.14±0.52	0.83±0.45	0.24±0.07
F	5.58	9.13	9.95	4.39	5.11
P-value	<0.05	<0.05	<0.05	<0.05	<0.05

Table III. Comparison of inflammatory cytokines between groups.

Group	TNF-α, ng/l	IL-6, pg/ml	MMP-9, ng/ml
Saline	1.45±0.87	113.45±15.37	77.37±13.22
Rosuvastatin	1.16±0.65	97.23±14.42	66.14±12.46
Low-dose demethylzeylasteral	0.86±0.51	90.15±12.36	45.05±9.67
High-dose demethylzeylasteral	0.72±0.46	76.28±9.76	34.16±7.45
F	7.67	5.47	4.79
P-value	<0.01	<0.01	<0.01

TNF-α, tumor necrosis factor-α; IL, interleukin; MMP-9, metalloproteinase-9.

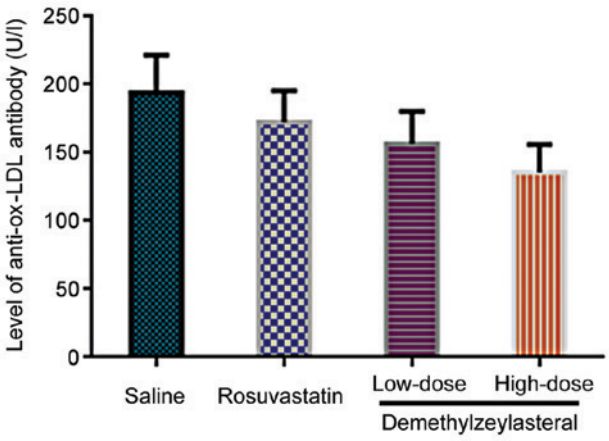

Figure 1. Comparison of autoimmune index between groups. ox-LDL, oxidized low-density lipoprotein.

Comparison of blood lipid levels. As shown in Table IV, the saline group, the rosuvastatin group, and the low-dose demethylzeylasteral group had significantly higher TG, TC, and LDL-C than the high-dose demethylzeylasteral group (P<0.01), however, their HDL-C content was significantly lower than the high-dose demethylzeylasteral group (P<0.01).

Evaluation of plaque lesions. The Sudan staining and H&E staining of the rabbit thoracic aortas after 30-days of treatments showed that the strips and the patches of the orange uplift on the arterial intimal surface in the saline group (Fig. 2), which indicated severe plaque lesions. In case of the rosuvastatin group endometrium, lumps or strips of uplift were found suggesting relatively light plaque lesions (Fig. 3), the low-dose demethylzeylasteral group showed relatively smooth intima

Table IV. Comparison of blood lipid levels between groups.

Group	TG, mmol/l	TC, mmol/l	HDL-C, mmol/l	LDL-C, mmol/l
Saline	1.11±0.11	3.22±0.32	0.47±0.06	0.83±0.12
Rosuvastatin	1.04±0.53	2.85±0.36	0.51±0.07	0.74±0.08
Low-dose demethylzeylasteral	1.05±0.46	2.97±0.41	0.54±0.05	0.77±0.13
High-dose demethylzeylasteral	0.93±0.52	2.63±0.52	0.63±0.11	0.62±0.12
F	6.43	6.07	5.42	6.15
P-value	<0.01	<0.01	<0.01	<0.01

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

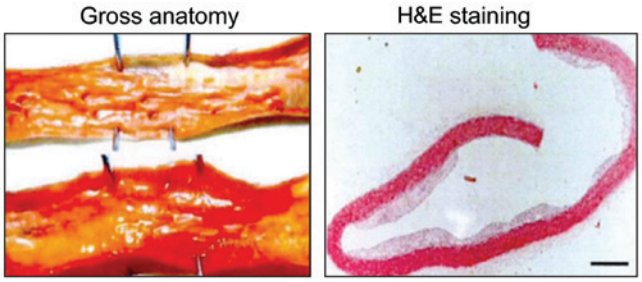

Figure 2. Sudan staining and haematoxylin and eosin (H&E) staining of rabbit thoracic aorta in the saline group (scale, 400 μm).

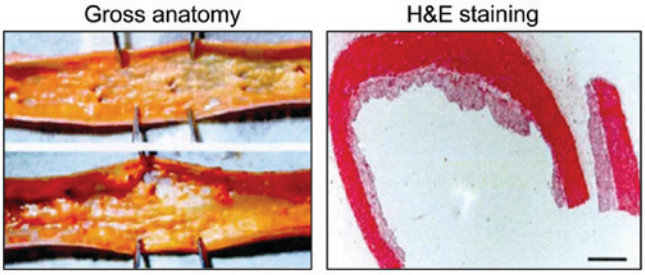

Figure 3. Sudan staining and haematoxylin and eosin (H&E) staining of rabbit thoracic aorta in the rosuvastatin group (scale, 400 μm).

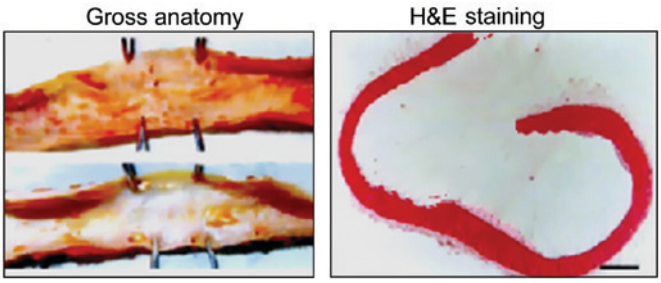

Figure 4. Sudan staining and haematoxylin and eosin (H&E) staining of rabbit thoracic aorta in the low-dose demethylzeylasteral group (scale, 400 μm).

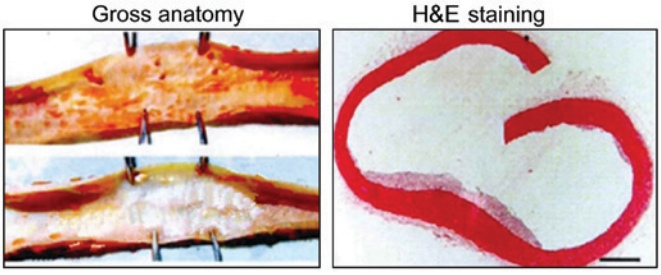

Figure 5. Sudan staining and haematoxylin and eosin (H&E) staining of rabbit thoracic aorta in the high-dose demethylzeylasteral group (scale, 400 μm).

with spotted uplift suggesting light plaque lesions (Fig. 4), and the smoothest intima and the lightest plaque lesions were found in the high-dose demethylzeylasteral group (Fig. 5).

Discussion

The immune system is activated in the process of AS formation, and the individual members of the immune system play an important role in AS (11). Studies have shown the existence of a large number of immune cells, for example, macrophages and lymphocytes, at the AS lesion sites (11-13). The macrophages can generate oxygen radicals and proteases, and can intake non-specific immune responses mediated by lipoprotein through scavenger receptors. In addition, macrophages can present exogenous antigens to T lymphocytes to initiate specific immune responses. Clinical studies discovered that lymphocytes accounted for 10-20% of nucleated cells in atherosclerotic plaques (13). Futhermore, lymphocytes are divided into T-lymphocytes, B-lymphocytes and NK cells, where T-lymphocytes ($CD3^+$) can further be divided into two main groups, namely, T help/inducer cells ($CD4^+$) and T suppressor/cytotoxic cells ($CD8^+$). It is found that during the development of atherosclerotic plaques, T lymphocytes enhance the local immune responses, promote phagocytosis of macrophages and secret cytokines and growth factors, and at the same time, it also promotes the proliferation, migration, and phagocytosis of medial vascular smooth muscle cells (14). Activated $CD4^+$ T lymphocytes produce a large amount of interferon-γ (INF-γ), TNF-α and other pro-inflammatory cells, and thus affect the metabolisms of the extracellular matrix and the functions of endothelial cells. The activation of $CD8^+$ T cells actually kills adjacent cells by physical contacts. In other words, all the medium produced in the process aggregates $CD8^+$ cells to kill smooth muscle cells and macrophages, which can promote the process of AS, and cause plaque ruptures and other complications (15,16). The activation of NK cells produce proinflammatory cytokines and promote AS. Humoral immunity was also involved in the formation

of AS. Normal artery walls had no immunoglobulins, while deposited immunoglobulins were found in AS lesions, which suggest that immunoglobulins were involved in the occurrence of AS. Complements are important components of the innate immunity. Products generated during complement activation are common effector molecules shared by specific immunity and nonspecific immunity, and play important roles in the body's resistance to infection, immune regulation, and immune surveillance, however, under certain circumstances, they can also cause tissue damage. It is found (17-20) that lesions of AS had not only complements depositions but also activated compliments resulting in damaged tissues.

Anti-inflammatory and anti-immune drugs include nonsteroidal anti-inflammatory drugs, cyclooxygenase-2 inhibitors, cyclosporine, methotrexate and traditional Chinese medicines, *Tripterygium* is considered the strongest immunosuppressive Chinese medicine, which have fewer side effects than western medicines (21). The plant *Tripterygium* belongs to the Celastraceae family and has been used as a pesticide for nearly two thousand years. Recently, it is found that *Tripterygium* has anti-inflammatory and immune suppression effects, and it has gradually been used for the treatment of autoimmune diseases, including rheumatoid arthritis, ankylosing spondylitis and systemic lupus erythematosus (22-25). Over 150 kinds of ingredients, mainly alkaloids, diterpenoids, triterpenoids, sesquiterpene and carbohydrates have been isolated from *Tripterygium* plants, including demethylzeylasteral (T96), a triterpene monomer, which is isolated from the root bark of *Tripterygium* by the Department of Pharmacy, Zhongshan Hospital of Fudan University. Compared with other currently known monomeric compounds isolated from *Tripterygium* such as triptolide, celastrol and *Tripterygium* glycosides, demethylzeylasteral demonstrated the strongest anti-immune effects and lowest toxicity. Note that the T cells are the central part of the entire immune response. The immune intervention targeted at T cells can effectively control the body's immune damage. *Tripterygium* could regulate T cells from many aspects. *Tripterygium* and its extracts could inhibit T cell proliferation. For instance, Xu *et al* (26) used mitogen ConA to stimulate splenocytes. The addition of T96 was found to be able to inhibit the transformation of spleen cells and the functions of T and B lymphocytes, and the increase in dose of T96 can enhance the degree of inhibition. Pan *et al* (27) studied PG490-88 (a semi-synthetic derivative of triptolide) on graft-versus-host disease (GVHD) and the *in vitro* experiments found that when $V\beta3^+$ cells were in the logarithmic amplification phase, PG490-88 could inhibit the expression of spleen $CD4^+$ $V\beta3^+$ and $CD8^+$ $V\beta3^+$ T cells and may thus inhibit the expansion of alloreactive T cells, thereby preventing the occurrence of GVHD. *Tripterygium* can also inhibit T cell proliferation induced by antigen, superantigen and mitogen by exerting its pharmacological effects by regulating $CD3^+$, $CD4^+$ and $CD8^+$ cells, and the $CD4^+/CD8^+$ ratio. Moreover, *Tripterygium* can inhibit the proliferation of B cells and the production of immunoglobulins, as well as the production of antibodies. It was also proposed (28) that *Tripterygium* could inhibit the expression of CD40 mediated by ionomycin and may thus inhibit the growth and differentiation of B cells and the class switching of immunoglobulins, and suppress the humoral immunity. Note that *Tripterygium* and its extracts can regulate a variety of cytokines in the body. Triptolide inhibits inflammation by inhibiting the generation of TNF-α and therefore the production of IL-6. It can also act on the transcription level of IL-2 and its receptor gene, inhibiting the expression of both of them. The transcription inhibition suppresses the immune responses mediated by IL-2 cells and thereby limit the clonal expansion of activated T cells. The Th1 cells mainly secrete INF-γ and other pro-inflammatory cytokines, while the Th2 cells secrete IL-4 and other anti-inflammatory factors. It is found that the inhibition by *Tripterygium* is stronger on Th2 than on Th1, which helps to maintain the ratio of Th1/Th2 and balance the pro-inflammatory anti-inflammatory functions. In addition, triptolide TZ93 significantly inhibits the activity of NK cells in the peripheral blood mononuclear cells after PHA-stimulation. It may inhibit lymphocyte activation like other substances, which influence the NK cell activity by inhibiting the secretion of IL-2 and the expression of IL-2 receptors (29,30). In summary, *Tripterygium* and its extracts can regulate the body's immune functions by affecting the proliferation and activation of T and B cells, adjusting the ratio of regulatory T cell subsets, modulating the production of immunoglobulins and NK cells, and influencing the secretion of various cytokines.

Our study showed that the saline group, the rosuvastatin group and the low-dose demethylzeylasteral group had significantly lower activated T lymphocyte parameters $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ ($P<0.05$), and significantly higher levels of sIL-2R, immunoglobulins IgG, IgA and IgM, complements C3 and C4, anti-ox-LDL antibody, TNF-α, IL-6 and MMP-9 ($P<0.01$) when compared with the high-dose demethylzeylasteral group. Moreover, TG, TC, LDL-C contents were found significantly lower and their HDL-C contents were significantly higher in high-dose demethylzeylasteral group ($P<0.01$) than the other three groups. Furthermore, Sudan staining and H&E staining of rabbit thoracic aortas after 30-day treatments showed that the high-dose demethylzeylasteral group had the smoothest intima, indicating the lightest plaque lesions among the four groups. These results suggest that AS is a systemic immune-mediated chronic inflammatory disease and the high dose of demethylzeylasteral is more effective in alleviating AS than low-dose demethylzeylasteral and rosuvastatin, and thus it may be considered as a suitable choice for anti-immunization therapy.

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