Effects of 3,4-Dihydroxyacetophenone on the Hypercholesterolemia-Induced Atherosclerotic Rabbits

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3,4-Dihydroxyacetophenone (3,4-DHAP) is one herbal extract from bald Mao-dong-qing leaves. We reported that 3,4-DHAP had anti-inflammatory function by decreasing tumor necrosis factor-α (TNF-α) secretion in macrophages. The aim of the study was to examine the effects of 3,4-DHAP on plasma and liver lipids, plasma alanine aminotransferase (ALT) and TNF-α level, vascular cell adhesion molecule-1 (VCAM-1) expression, plaque vulnerability and vascular inflammation in hypercholesterolemia-induced atherosclerotic rabbits. Male New Zealand white rabbits were randomized into negative control, positive control, 3,4-DHAP and simvastatin groups. From weeks 2 to 12, the rabbits were treated with 3,4-DHAP or simvastatin. At weeks 12, all the animals were sacrificed. Plasma lipids and ALT were measured using the enzymatic endpoint method. Plasma TNF-α was measured using enzyme-linked immuno sorbent assay (ELISA). Liver lipids concentrations were estimated using commercial kits. The expression of VCAM-1 was measured using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Histological analysis was used to evaluate the pathologic changes of rabbit aortas. The results showed that 3,4-DHAP markedly lowered plasma and liver lipids, lowered plasma ALT and TNF-α levels compared with the positive control group. VCAM-1 mRNA and protein were markedly inhibited by 3,4-DHAP. Decreased aortic plaque instability was evident in 3,4-DHAP-treated rabbits, as demonstrated by a thickened elastic layer, increased vascular smooth muscle cells (VSMCs) accumulation in the plaques, less neointima hyperplasia and macrophages recruitment. 3,4-DHAP may attenuate the progression of atherosclerotic lesions and stabilize plaques by lowering plasma lipids, the number of macrophages and the expression of VCAM-1, while increasing the number of VSMCs in the atherosclerotic plaques.

Key words 3,4-dihydroxyacetophenone; vascular cell adhesion molecule-1; atherosclerosis

Atherosclerosis is an inflammatory disease. Atherosclerosis associated with the rupture of vulnerable plaque is the main cause of cardiovascular events. The characteristics of vulnerable plaque include a large lipid core, thin fibrous cap, inflammation and immune cell activation. Growing evidence shows that macrophages play a pivotal role in all stages of atherosclerosis. Vascular cell adhesion molecule-1 (VCAM-1) can strengthen the adhesion of leukocytes to vascular wall, promote leukocytes entering intima, transforming into macrophages. Evidence is accumulating that low-density lipoprotein cholesterol (LDL-C) is the principal risk factor for atherosclerosis, and it is identified as the primary target for cholesterol-lowering therapy. Tumor necrosis factor-α (TNF-α) is one of the most important mediators of inflammation and may mediate endothelial dysfunction.

3,4-Dihydroxyacetophenone (3,4-DHAP) is extracted from herb; clinical experiments and pharmacological studies of it have been on for many years in coronary heart disease, pregnancy-induced hypertension syndrome and so on. Our previous studies have found that 3,4-DHAP had the anti-inflammatory effect by inhibiting the secretion of TNF-α at mouse macrophage cells (RAW264.7 cell line). However, little is known about the effects of 3,4-DHAP on atherosclerotic progress.

The hypercholesterolemia-induced atherosclerotic rabbit model has been widely used in studies of cardiovascular diseases. Here, we report that 3,4-DHAP lowers plasma and liver lipids, lowers plasma alanine aminotransferase (ALT) and TNF-α levels, attenuates aortic plaque vulnerability by lowering the number of macrophages and the expressions of VCAM-1 mRNA and protein, increasing the number of vascular smooth muscle cells (VSMCs) in a hypercholesterolemia-induced atherosclerotic rabbit model.

MATERIALS AND METHODS

Hypercholesterolemia-Induced Model The present study conformed to the national guidelines on the care and use of laboratory animals. The animal experiments were approved by the ethics committee of Weifang Medical College. Thirty-two male New Zealand white rabbits (aged 12 weeks, weighing 2.3–2.4 kg) were obtained from the animal center of Weifang Medical College. After an adapted period (one week) with standard diet, blood was collected after overnight fasting from the rabbit auricular veins to obtain basal lipids. The rabbits were divided into four groups (8 rabbits/group) based on blood lipid levels and body weights: negative control group, 3,4-DHAP and simvastatin group. The rabbits in the negative control group were fed a standard diet; those in other groups were fed a hypercholesterolemic diet (containing 1% cholesterol, 5% axungia porci and 7.5% yolk powder) for 12 weeks. Each rabbit was fed with fresh rabbit food of 120g daily. Water was supplied ad libitum. From weeks 2 to weeks 12, the rabbits were injected the samples one time daily; the rabbits in the positive control group were intraperitoneally injected equal dose 1% ethanol, the rabbits in 3,4-DHAP group were intraperitoneally injected 3,4-DHAP (10mg/kg, TCI, TC1).
Japan), the rabbits in simvastatin group were intraperitoneally injected simvastatin (10 mg/kg, Nanjing Debiochem Co., Ltd., China). 3,4-DHAP was dissolved in ethanol, filtrated and stored avoiding light at 4°C. When using, heat to 37°C, dilute the solution using double-distilled water. According to previously described, simvastatin was dissolved in ethanol, NaOH (1 mol/L) was added to the solution, and then incubated at 50°C for 2 h. The pH was adjusted to 7.0–7.2 using 0.1 mol/L HCl, and then dilutes the solution using double-distilled water. Simvastatin solution was filtrated and stored at 4°C. When using, heat to 37°C. Body weights, blood lipids, ALT and TNF-α of the animals were recorded monthly. At the end of weeks 12, all rabbits were sacrificed. Aortas were excised for assessment of atherosclerotic changes, determinations of VCAM-1 mRNA and protein expressions and histological immunostaining.

Plasma Lipids, Liver Lipids and Plasma ALT Measurements At weeks 0, 4, 8 and 12, blood samples were obtained from the rabbit auricular veins, then centrifuged to obtain sera in a total volume of 25 µL. Plasma concentrations of TC, TG, HDL-C, LDL-C and ALT were measured using rabbit high-sensitive ELISA kits (Sangon Biotech Co., Ltd., China). The plasma concentration of TNF-α was estimated using commercial kits (Sangon Biotech Co., Ltd., China). The lipids in the liver were extracted, and then purified with chloroform and methanol (2 : 1, v/v). The extracts were dissolved in chloroform, TC and TG concentrations were measured using rabbit high-sensitive ELISA kits (Sangon Biotech Co., Ltd., China). The reaction was performed in T3 Thermocycler (Bio-Rad, U.S.A.). Secondary antibody was used (peroxidase-labeled, anti-mouse horseradish peroxidase-labeled immunoglobulin G (IgG) antibody (Sangon Biotech Co., Ltd., China, 1 : 200) according to elastic fibres staining kit (Beijing Yili Chemicals Co., Ltd., China) instructions for elastic layer thickness analysis. Simvastatin solution was filtrated and stored with chloroform and methanol (2 : 1, v/v). The extracts were dissolved in chloroform, TC and TG concentrations were estimated using commercial kits (Sangon Biotech Co., Ltd., China).

Evaluation of Plasma TNF-α by Enzyme-Linked Immunosorbent Assay (ELISA) The plasma concentration of TNF-α were measured using rabbit high-sensitive ELISA kits (Sangon Biotech Co., Ltd., China) according to the manufacturer’s instructions. Absorbance at 450 nm was provided with VERSA microplate reader (U.S.A.). A standard curve was generated from standards of known concentration. Values of samples were calculated from the standard curve.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) After 12 weeks of treatment, total mRNA was extracted from the rabbit aortas using TRIZOL reagent (Invitrogen Inc., MD, U.S.A.) according to manufacturer’s instructions. cDNA was synthesized from 1 µg total mRNA using the M-MLV reverse transcriptase (Sangon Biotech Co., Ltd., China). The reaction was performed in T3 Thermocycler (Biometra, Germany) at 42°C for 1 h, and then the enzyme was denatured at 70°C for 10 min. PCR amplification was performed in a total volume of 25 µL containing 1 µL template cDNA. Primers’ sequences are shown in Table 1. PCR products were analyzed by gel electrophoresis (2% agarose gel). The gene bands were photographed under UV transillumination. The gene expression was semi-quantitated after normalizing with glyceraldehyde-phosphate dehydrogenase (GAPDH) gene. Analysis of the data was performed using Gel-pro32.exe software (Media Cybernetics, U.S.A.).

Results

Plasma Lipids, Liver Lipids and Plasma ALT Alteration in TC, TG, HDL-C, LDL-C concentrations in all four groups was found at 4°C. When using, heat to 37°C, dilute the solution using double-distilled water. Simvastatin solution was filtrated and stored with chloroform and methanol (2 : 1, v/v). The extracts were dissolved in chloroform, TC and TG concentrations were estimated using commercial kits (Sangon Biotech Co., Ltd., China).

Table 1. Primers for the Target Genes

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primers</th>
<th>Accession No.</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>Sense 5′-GCTGTGATCCCCAACATT-3′</td>
<td>AY212510</td>
<td>55</td>
<td>453</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5′-GAGCTGAACGGGGAGAACACAT-3′</td>
<td>NM-001082253</td>
<td>63</td>
<td>464</td>
</tr>
</tbody>
</table>

Rabbits Aortas Homogenate and Western Blot Analysis Rabbits’ aortas were cut into pieces and homogenized in radioimmunoprecipitation assay lysis buffer (1 mL/100 g tissue, Sangon Biotech Co., Ltd., China) as previously reported. VCAM-1 protein (primary antibody, 1 : 2000, Santa Cruz Biotechnology, U.S.A.) was characterized by Western blot, GAPDH protein was used as internal control (monoclonal antibody against GAPDH, 1 : 1000, Santa Cruz Biotechnology, U.S.A.). Secondary antibody was used (peroxidase-labeled, 1 : 20000, Roche Applied Science, IN, U.S.A.). The chemiluminescence was used to develop the bands. Quantification was performed with Gel-pro32.exe software (Media Cybernetics, U.S.A.).

Histological Analysis The aortic artery tissues were fixed in formalin and paraffin-embedded. Three-micrometer sections obtained from each vessel at 5-mm intervals were used for histological analysis. Cryosections (10 µm) were made for elastic layer thickness analysis.

Intima-Media and Elastic Layer Thickness Sections (3 µm) were stained with hematoxylin and eosin for intima-media thickness analysis. Cryosections (10 µm) were stained according to elastic fibres staining kit (Beijing Yili Chemicals Co., Ltd., China) instructions for elastic layer thickness analysis. Firstly, stained for 10 min, rinsed with distilled water; secondly, differentiated; thirdly, stained with counterstain for 10 s, then mounted using neutral balsam.

Immunohistochemistry Macrophages and VSMCs in atherosclerotic lesions were evaluated using immunohistochemical analysis. Sections were incubated with 3% hydrogen peroxide for 10 min, with 5% bovine serum albumin serum for 20 min, with mouse monoclonal anti-rabbit macrophages RAM-11 (DAKO, U.S.A., 1 : 300) antibody or mouse monoclonal antibodies against α-smooth muscle actin (α-SM actin, Abcam, U.S.A., 1 : 100) for 2 h at 37°C, and with goat-anti-mouse horseradish peroxidase-labeled immunoglobulin G (IgG) antibody (Sangon Biotech Co., Ltd., China, 1 : 200) for 30 min at 37°C. A DAB Kit (Boster, China) was used to visualize antibody binding, according to manufacturer’s instructions. The sections were visualized under Olympus BX50 microscope. The analyses were performed microscopically in conjunction with Image Pro-plus 6.0 software (Media Cybernetics, U.S.A.) by an observer blinded to the study.

Statistical Analysis All data were expressed as the mean±S.D. SPSS 18.0 was used for all the analyses. Significant differences among the groups were determined by one-way ANOVA test. Values of p<0.05 were taken as statistically significant.

Results

3,4-DHAP Treatment Regulates Plasma Lipids, Liver Lipids, Plasma TNF-α and Body Weight Alteration in TC, TG, HDL-C, LDL-C concentrations in all four groups was found at 4°C. When using, heat to 37°C, dilute the solution using double-distilled water. Simvastatin solution was filtrated and stored with chloroform and methanol (2 : 1, v/v). The extracts were dissolved in chloroform, TC and TG concentrations were estimated using commercial kits (Sangon Biotech Co., Ltd., China).
Fig. 1. 3,4-DHAP Treatment Regulates Plasma Lipids
(A) Levels of plasma total cholesterol at weeks 0, 4, 8 and 12 in rabbits. (B) Levels of plasma triglyceride (TG) at weeks 0, 4, 8 and 12 in rabbits. (C) Levels of plasma LDL-C at weeks 0, 4, 8 and 12 in rabbits. (D) Levels of plasma HDL-C at weeks 0, 4, 8 and 12 in rabbits. n=8. *p<0.05 vs. PC group; **p<0.001 vs. PC group; *p<0.05 vs. simvastatin group.

Fig. 2. 3,4-DHAP Treatment Decreases Plasma ALT, TNF-α Levels and Liver Lipids
n=8. *p<0.05 vs. PC group; **p<0.001 vs. PC group; *p<0.05 vs. simvastatin group.
were shown in Fig. 1. At weeks 4, 8 and 12, in positive control group, TC, TG, HDL-C and LDL-C concentrations were increased significantly compared to negative control group \((p<0.001)\). At weeks 4, 3,4-DHAP markedly decreased TC \((p<0.05)\) and TG \((p<0.001)\) in comparison with positive control group, decreased TG \((p<0.001)\) in comparison with simvastatin group. At weeks 8 and 12, 3,4-DHAP markedly decreased TC, TG, LDL-C and HDL-C \((p<0.001)\) compared to positive control group, and significant differences were observed between 3,4-DHAP and simvastatin groups by TC \((p<0.05)\), TG, LDL-C and HDL-C \((p<0.001)\). At weeks 8 and 12, in positive control group, ALT level was markedly increased compared to negative control group \((p<0.001)\). At weeks 8 and 12, 3,4-DHAP markedly decreased ALT level compared to positive control \((p<0.001)\) and simvastatin group \((p<0.05)\) (Fig. 2A).

The liver lipids were shown in Figs. 2C, D. At weeks 4, 8 and 12, 3,4-DHAP markedly decreased TC and TG in the liver compared to positive control, but no significant differences were observed between 3,4-DHAP and simvastatin groups \((p>0.05)\).

To assess the anti-inflammatory effect of DHAP, the plasma TNF-\(\alpha\) level was detected using ELISA (Fig. 2B). At weeks 4, 8 and 12, in positive control group, TNF-\(\alpha\) level was markedly increased compared to negative control group \((p<0.001)\); 3,4-DHAP markedly decreased TNF-\(\alpha\) level compared to positive control \((p<0.001)\), and no significant differences were observed between 3,4-DHAP and simvastatin group \((p>0.05)\).

At the beginning of this study, the body weight of all experimental rabbits ranged from 2.3 to 2.4 kg. All the rabbits completed the experimental process. At weeks 4, 8, 12, all rabbits gained weight, the final weight ranged from 2.9 to 3.1 kg, but no significant difference was observed at any time point (data not shown).

**3,4-DHAP Treatment Regulates Aortic VCAM-1 Gene and Protein Expressions** 3,4-DHAP significantly decreased VCAM-1 mRNA and protein expressions in rabbit aortic arteries compared to positive control animals \((p<0.001)\). Significant difference was observed between 3,4-DHAP and simvastatin group \((p<0.05)\) in gene and protein expressions (Fig. 3). These data suggest that 3,4-DHAP attenuates carotid plaque vulnerability by decreasing VCAM-1 expression.

**3,4-DHAP Treatment Stabilized Aortic Plaque and Decreased Vascular Inflammation** Our results (Figs. 4-1, -2) showed that neointima hyperplasia was significantly inhibited by 3,4-DHAP, compared with the positive control group \((p<0.001)\). In contrast, 3,4-DHAP stabilized aortic plaque by increasing the thickness of the elastic layer, compared to the positive control group \((p<0.001)\). The plaque content of VSMCs was markedly increased in positive control group, but lower than that in 3,4-DHAP group \((p<0.001)\). In addition, compared with the positive control group, 3,4-DHAP significantly inhibited vascular inflammation by decreasing macrophage infiltration into the wall of aorta \((p<0.001)\); Significant difference was observed between 3,4-DHAP and simvastatin group by intima-media thickness \((p<0.05)\), elastic layer thickness \((p<0.001)\) and macrophage accumulation \((p<0.05)\).

**DISCUSSION**

In our study, we found that 3,4-DHAP treatment for 12 weeks suppressed TC, TG, LDL-C, ALT and TNF-\(\alpha\) levels,
reduced the liver lipids, down-regulated VCAM-1 mRNA and protein expressions in rabbit aortic plaques, and reduced plaque vulnerability. The findings indicate that 3,4-DHAP may attenuate atherosclerosis progression in hypercholesterolemic rabbits by interfering with the inflammatory pathway and affecting lipid parameters.

**Effects of 3,4-DHAP on Blood Circulation System**

3,4-DHAP is the effective ingredients of bald Mao-dong-qing leaves (*Ilex pubescens* Hook. *et Arn.* var. *glabra* CHANG), its formula is $C_{8}H_{8}O_{3}$; the relative molecular mass is 152u. Previous studies showed that 3,4-DHAP could dose-dependently inhibit the release of thromboxane A$_{2}$ (TXA$_{2}$) from rabbit platelet aggregate induced by collagen or arachidonic acid (AA) either *in vitro* or *in vivo*, reduce the product of prostacyclin (PGI$_{2}$) in largely dose, therefore, regulate the balance of TXA$_{2}$/PGI$_{2}$. It showed that 3,4-DHAP had the stronger inhibitory effect on plasminogen activator inhibitor (PAI) than that on tissue plasminogen activator (tPA) related to the dosage, then induce the ratio of PAI/tPA declining, therefore, it was considered that inhibition of thrombus by 3,4-DHAP might be related to its regulating effect on balance of PAI/tPA of vascular endothelial cells. 3,4-DHAP might relax pulmonary vessels by regulating the ratio of cAMP/cyclic guanosine monophosphate. 3,4-DHAP could regulate the balance of nitric oxide/endothelin which might be a mechanism of treating pregnancy induced hypertension syndrome.

**3,4-DHAP Treatment Regulates Lipid and Stabilizes Carotid Plaques, and Decreases Vascular Inflammation**

Our precious results showed that 3,4-DHAP (10$^{-7}$mol/L) had anti-inflammatory effects, could elevate hemeoxygenase-1/carbon monoxide expression, effectively decrease the secretion of TNF-α induced by LPS in mouse macrophages (RAW264.7 cell lines). In this study, 3,4-DHAP could decrease the plasma TNF-α levels. In ApoE(−/−) mice, 3,4-DHAP treatment (20mg/kg) significantly lowered total cholesterol, TG, LDL-C levels. However, there were no significant changes in serum HDL-C levels. In this study, we found that 3,4-DHAP treatment decrease TC, TG, LDL-C levels, and lipid deposition (data not shown) in the aortas and livers in hypercholesterolemia-induced atherosclerotic rabbits. High LDL-C levels play a crucial role in the vascular events. Numerous clinical trials have shown the efficacy of lowering LDL-C for reducing Coronary heart disease (CHD) risk. It has been suggested that each percentage reduction in LDL-cholesterol can reduce CHD risk by 2%.

What are the mechanisms behind the cholesterol-lowering effects of 3,4-DHAP? In our study, the basal cholesterol levels
in rabbits are very low; the cholesterol levels in rabbits with 3,4-DHAP treatment are lower than in rabbits without treatment. At weeks 8 and 12, the cholesterol-lowering effects of 3,4-DHAP is better than that of simvastatin. Simvastatin, a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, could decrease cholesterol synthesis and enhance removal of LDL cholesterol by the LDL receptor pathway in hepatocytes. Could 3,4-DHAP suppress cholesterol uptake or reduce circulating cholesterol levels in these rabbits? It’s unclear. Many effects of statins are independent of cholesterol-lowering functions, for example, stabilizing plaque and decreasing vascular inflammation. Zhou et al. reported that atorvastatin could reduce plaque vulnerability altering the 5-lipoxygenase pathway. It’s still unclear of 3,4-DHAP.

In reverse cholesterol transport, HDL collects peripheral cholesterol, and transport cholesterol to the liver for excretion bile. Many evidences prove that HDL plays an important role in atherogenesis. A low level of HDL-C increases the risk for CHD and increases mortality. However, some evidences weaken the hypotheses that HDL-C level relate directly to CHD status and that elevating HDL-C is necessary. In our study, we found 3,4-DHAP could decrease the plasma HDL-C level, but 3,4-DHAP exhibits an anti-atherosclerotic effect. We suppose that promoting HDL2b transform into HDL3a and increasing the expression of SR-BI in the liver may be the lowering-HDL-C mechanism of 3,4-DHAP.

A high plasma TG level was associated with increased coronary disease risk. Peroxisome proliferators-activated receptor (PPAR)-α activity could reduce TG level by increasing β-oxidation of fatty acids, increasing lipoprotein lipase to promote lipid into the cell, and suppressing apoC-III, which disturbs the clearance of TG-containing lipoproteins. We propose that 3,4-DPAH decreases the plasma TG level by increasing the activation of PPAR-α, this hypothesis need to be further proved.

Fig. 4-2. (B) Measurement of Aortic Intima-Media Thickness between Groups in Rabbits, (C) Thickness of Elastic Layer between Groups in Rabbits, (D) Level of SMC in Aortic Plaques between Groups in Rabbits and (E) Level of Macrophage in Aortic Plaques between Groups in Rabbits

Mean density=integrated optical density/area. D=3,4-DHAP; S=simvastatin; PC= positive control; NC= negative control, n=8. Bar=10µm. **p<0.001 vs. PC group; #p<0.05 vs. simvastatin group; ##p<0.001 vs. simvastatin group.
macrophages is associated with decreased VCAM-1 expression, higher VSMC content in the plaque. \(^{28-30}\) Our data show that the effects of 3,4-DHAP are similar to simvastatin, decreasing the expressions of VCAM-1 mRNA and protein and the number of macrophages, and increasing the number of VSMCs in rabbit aortic plaques. However, it is not clear whether 3,4-DHAP may induce stabilized aortic plaque and decrease vascular inflammation via lipid-lowering dependent or independent effects.

In conclusion, 3,4-DHAP may attenuate the progression of atherosclerotic lesions by lowering the plasma lipids level, the number of macrophages and the expressions of VCAM-1 mRNA and protein, and increasing the number of VSMCs in the plaques. However, the exact mechanisms of these beneficial effects are unclear, thus warranting further studies.

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**REFERENCES**
