

Original Research Article

Dioscorea deltoidei (Dioscoreaceae) leaf extract exerts anti-atherosclerotic effect in rats via down-regulation of phosphorylated JAK/STAT

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Abstract

Purpose: To investigate the effect of leaf extract of *Dioscorea deltoidea* (Dioscoreaceae) leaf (DDE) on atherosclerosis-induced aorta wall damage in a rat model, and the underlying mechanism of action.

Methods: Rats were fed high-fat diet containing vitamin D2 for 16 weeks to induce atherosclerosis. Histopathological changes in the aorta were examined using hematoxylin and eosin (H & E) staining, while ELISA kits were used to measure cytokine levels.

Results: Treatment with DDE significantly ($p < 0.05$) alleviated atherosclerosis-induced increase in mean lesion area in the rat aorta. The mean lesion area in atherosclerotic rats was decreased to 51.5, 21.2 and 2.3 mm², on treatment with DDE at doses of 2.5, 5 and 10 mg/kg, respectively. Furthermore, DDE significantly suppressed atherosclerosis-induced elevation in IL-1 β and IL-6 levels in the rat aorta ($p < 0.05$). The levels of MCP-1 and TNF- α decreased in the atherosclerotic rats on treatment with DDE. In DDE-treated rats, the atherosclerosis-induced increase in the levels of Ang II, AT1, AT2, p-STAT3, p-p65 and p-p38 were significantly decreased, relative to the model group ($p < 0.05$). However, DDE treatment did not alter the levels of total STAT3, p65 and p38 in the rat aorta tissues.

Conclusion: These results indicate that DDE inhibits inflammatory response and atherosclerosis-induced damage to aorta wall. Moreover, RAAS expression, inflammatory cytokines and JAK/STAT signalling pathway were down-regulated in atherosclerotic rats on treatment with DDE. Thus, DDE may be a potential source of drug for the management of atherosclerosis.

Keywords: Atherosclerosis, *Dioscorea deltoidei*, Inflammation, Aorta damage, Angiotensin II

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INTRODUCTION

Atherosclerosis, a common pathological condition, is the leading cause of cerebrovascular and coronary diseases associated with very high morbidity and mortality globally [1]. Atherosclerosis is characterized by formation of fatty streaks and accumulation of

atheromatous plaques [2]. The primary method of treatment of atherosclerosis is lifestyle modification, whereas medicines are used for advanced stage of the disease [3]. The most common class of medicines used for the treatment of atherosclerosis are statins [3]. However, the currently-available medicines are ineffective for suppression of atherosclerosis in

most of the susceptible individuals [4]. This indicates that more effective drugs are required for treating atherosclerosis. Abnormality in metabolism of lipids is considered to be a major factor in induction of atherosclerosis [5]. Individuals with similar cholesterol levels have been found to show discrepancies in the pathogenesis of atherosclerosis [6].

Evidence from various studies have demonstrated the key role of inflammation in the pathogenesis of atherosclerosis [5,6]. Initially, it was found that atherosclerosis was associated with levels of macrophages, but later-on, leukocytes were also found to be associated with development of atherosclerotic lesions [7]. Cytokines give rise to chronic inflammation [8]. The signalling molecules associated with inflammation include nuclear factor- κ B (NF- κ B) and Janus kinase/signal transducers and activators of transcription (JAK/STAT). In addition, the mitogen-activated protein kinase (MAPK) pathway is involved in chronic inflammation [8]. Studies on cytokine-deficient animals have revealed the key role of interleukins and apolipoprotein E in the pathogenesis of atherosclerosis [9]. Indeed, mice deficient in interleukin-1 β had reduced atherosclerotic lesions, when compared with mice that expressed interleukin-1 β [9].

Cardiovascular risk has been linked to levels of C-reactive protein, TNF- α and IL-6. Atherosclerosis is also associated with the expression of renin-angiotensin-aldosterone system (RAAS). The main effector of RAAS is angiotensin II (AngII) and it plays important role as a proinflammatory factor. In an earlier study, an increase in anti-inflammatory effect was observed when the expression of AngII was blocked using ACEIs and ARBs [9].

Dioscorea genus belongs to the Dioscoreaceae family which grows at high temperatures in tropical and sub-tropical regions globally. These plants have been used for pharmacological applications as formulations, and for industrial uses [10]. The tubers of several *Dioscorea* species have been used to treat several diseases/ailments such as cough, cold, stomach ache, leprosy, burns, fungal infections, dysentery, skin diseases, rheumatism and arthritis [10]. Several pharmaceutically important compounds have been isolated from plants of *Dioscorea* species [11].

In the present study, the effect of leaf extract of *Dioscorea deltoidea* (DDE) on atherosclerosis-induced aorta wall damage in vivo was investigated in a rat model.

EXPERIMENTAL

Animals

Seventy-five male, 12-week-old Sprague Dawley rats weighing 210 - 240 g, were obtained from the Animal Centre of the Binzhou Medical University, China. The rats were kept at ambient temperature (23 ± 1 °C) under 12-h/12-h light/dark cycle and relative humidity of 62-65 %. Standard rodent diet and tap water were made available to the rats *ad libitum*. The Animal Ethics Committee of the Hubei University of Medicine, China approved all experimental protocols involving animal studies (approval no. EC/2018/02/00254); furthermore, the studies followed international guidelines for animal studies [12]. The rats were randomly assigned to five groups viz: sham, model (atherosclerotic) group, 2.5 DDE group (treated with DDE at a dose of 2.5 mg/kg), 5 DDE (treated with DDE at a dose of 5 mg/kg), and 10 DDE (given DDE at a dose of 10 mg/kg).

Atherosclerosis rat model

The atherosclerotic rat model was established by feeding the rats with high fat diet containing vitamin D2 for 16 weeks, as described earlier [13]. The high fat diet consisted of normal feed (83.3 %), chleolate (0.5 %), cholesterol (3 %), white plantation sugar (5 %), propylthiouracil (0.2 %), and lard (8 %). All rats were injected with vitamin D2 at a dose of 3×10^5 U/kg body weight at the beginning of the study. *Dioscorea deltoidea* leaf extract was given to the treatment groups from 5th week of the study via gastric perfusion.

Histopathological assessment

After 16 weeks, all rats were fasted for 8 h, after which they were anesthetized using 10 % chloral hydrate solution (0.3 ml/100 g). The rats were sacrificed via decapitation. The aortas were excised and sliced into sections which were used for determination of pathological changes. One portion was processed into histological slides and subsequently stained using oil red dye for demonstration of presence of lipids. Another portion of aorta was fixed in 4 % paraformaldehyde, sliced into thin sections, stained using hematoxylin and eosin (H&E), and examined under a light microscope.

Enzyme-linked immunosorbent assay (ELISA)

Inflammatory cytokine levels in the rat aorta tissues were measured using ELISA. Commercially available ELISA kits (R&D

Systems, Minneapolis, MN, USA) were used to measure the levels of MCP-1 (MJE00), IL-1 β (RLB00), IL-6 (R6000B), and TNF- α (RTA00) in the aorta tissues, in line with the kit protocols.

Immunohistochemical analysis

Aorta specimens embedded in paraffin were sliced into 3- μ m cross-sections using a microtome, de-paraffinized, and subsequently rehydrated. Citrate buffer (0.1 M; pH 6.0) was added to the slides, followed by heating for 25 min in a microwave oven. The activities of endogenously generated peroxidases were quenched by treatment with 3 % H₂O₂ for 30 min. Sections treated with BSA (2 %) were incubated overnight at 4 °C with anti-bodies against AT2, AngII and AT1. Thereafter, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies for 50 min. Staining and subsequent counterstaining were performed with diaminobenzidine and Harris hematoxylin, respectively. Semi-quantitative assessment of the expression levels was conducted on 0 - 3 scale.

Western blotting

Total protein was extracted from aorta tissues via homogenization in RIPA lysis buffer (Beyotime, Haimen China). The lysate was centrifuged at 12,000 x g for 25 min at 4 °C, and the protein content of the supernatant was estimated using BCA kits (Beyotime, Haimen China). Equal amounts of protein (50- μ g samples) were resolved on 10 % SDS-PAGE, and electro-transferred onto PVDF membranes. The membranes were blocked by incubation with 5 % skim milk/TBST for 1 h. Then, the membranes were incubated overnight at 4 °C with primary anti-bodies against p65, p-p65, p-STAT3, STAT3, p38 and p-p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Thereafter, the membranes were washed with PBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. BeyoECL Star (Beyotime Institute of Biotechnology) was used for

visualization. Quantification of protein bands was performed using GeneTools software, version 4.1 (Cambridge, UK).

Quantitative polymerase chain reaction (PCR)

Total RNA was isolated from aorta tissues using Ultrapure RNA kit. The RNA was reverse-transcribed to cDNA, followed by quantitative-PCR reaction using ABI-7900 System (Applied Biosystems, Inc.) and SYBR Green (Applied Biosystems, Inc.). The conditions used for amplification were: 94 °C for 8 min, then 48 cycles at 94 °C for 25 sec, followed by 58 °C for 20 sec. The mRNA levels were quantified for each gene, followed by calculation of relative expressions using the 2- $\Delta\Delta$ Ct method. The primer sequences used are shown in Table 1.

Statistical analysis

Data are expressed as mean \pm standard deviation (SD). Comparisons were done using one-way analysis of variance (ANOVA) and Bonferroni multiple comparison test. The GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for all statistical analyses. Differences were taken as statistically significant at $p < 0.05$.

RESULTS

DDE inhibited atherosclerosis

There was significant increase in average lesion area in DDE-treated rat group, when compared to the sham group ($p < 0.05$). As shown in Table 2, the average lesion area in the model atherosclerosis rats increased to 65.3 mm², relative to 1.8 mm² in the sham group. However, treatment with DDE led to significant reduction in atherosclerosis-induced increase in average lesion area in the rat aortas ($p < 0.05$). The average lesion area in atherosclerotic rats was decreased to 51.5, 21.2 and 2.3 mm², on treatment with DDE at doses of 2.5, 5 and 10 mg/kg, respectively.

Table 1: Sequences of primers used for RT-PCR

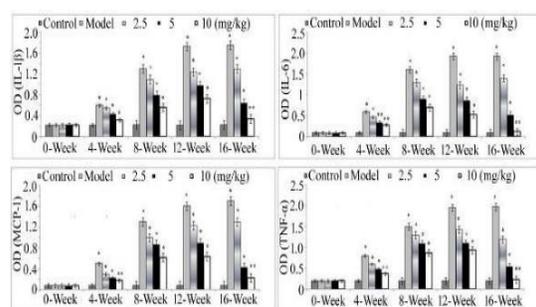
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
AT1	GCAGCACTTCACTACCAAATGG GC	CAGGACAAAAGCAGGCTAGGGAG A
AngII	CCGCATTTAACTGCTCACACA	ATCATGTAGTAGAGAACAGGAATT GCTT
AT2	GGAAGGTAGAACATACATTAATAA TG	AGAGAAACAGCAGCTAAAGAATT
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

Table 2: Effect of DDE on lesion areas in atherosclerosis rat model.

Group	Atherosclerotic area (mm ²)	Lipidoses area (mm ²)
Sham	1.8 ± 0.54	0.93 ± 0.32
Model	65.3 ± 5.43	45.5 ± 4.76
2.5 mg/kg	51.5 ± 5.27	34.6 ± 4.11
5 mg/kg	21.2 ± 3.87	12.9 ± 2.32
10 mg/kg	2.3 ± 0.42	1.8 ± 0.26

DDE down-regulated atherosclerosis-induced inflammatory cytokines

Atherosclerosis induction caused significant increases in levels of IL-1 β and IL-6 in rat aorta tissues, relative to the sham group ($p < 0.05$; Figure 1). Moreover, atherosclerosis increased the expression levels of MCP-1 and TNF- α in the rat aorta tissues. However, treatment with DDE at doses of 2.5, 5 and 10 mg/kg significantly suppressed the atherosclerosis-induced up-regulation in expression levels of IL-1 β and IL-6 in the rat aortas. Moreover, the expression levels of MCP-1 and TNF- α in rats with atherosclerosis were reduced on treatment with DDE.

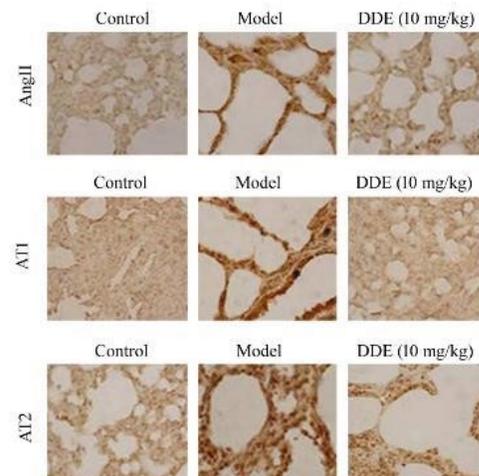
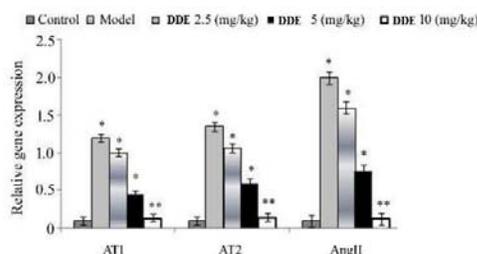
**Figure 1:** Effect of DDE on inflammatory cytokine levels. After induction of atherosclerosis, the rats were treated with DDE at doses of 2.5, 5 and 10 mg/kg. ELISA was used for determination of cytokine levels in the rat aortas. * $P < 0.05$, ** $p < 0.02$, compared to sham group

DDE decreased activation of atherosclerosis-induced RAAS

Atherosclerosis increased the expression levels of AngII, AT1 and AT2 in rat aorta tissues, when compared to the control group (Figure 2). However, treatment of the rats with DDE led to marked reversal of the atherosclerosis-induced increases in levels of AngII, AT1 as well as AT2 ($p < 0.05$). The reductions in levels of AngII, AT1 and AT2 were maximum in rats treated with DDE at a dose of 10 mg/kg.

Results from RT-PCR showed that atherosclerosis caused increases in mRNA expression levels of AngII, AT1 and AT2 in the

rat aorta tissues, when compared to the control group. However, DDE treatment significantly reversed atherosclerosis-induced increases in mRNA levels of AngII, AT1 and AT2. The results showed that DDE was most effective at the dose of 5 mg/kg.

**Figure 2:** Effect of DDE on RAAS activation. After induction of atherosclerosis, the rats were treated with DDE at doses of 2.5, 5 and 10 mg/kg. The mRNA levels of RAAS family members in the rat aortas were determined using immunohistochemical staining**Figure 3:** Effect of DDE on levels of RAAS members. After induction of atherosclerosis, the rats were treated with DDE at doses of 2.5, 5 and 10 mg/kg, followed by determination of mRNA expression levels of AngII, AT1 and AT2 mRNA. * $P < 0.05$, ** $p < 0.02$, compared to sham group

DDE alleviated atherosclerosis-induced inflammatory pathways

Atherosclerosis induction in rats led to up-regulated expression levels of p-STAT3, p-p65 and p-p38 in aorta tissues, when compared to the control group (Figure 4). However, atherosclerosis had no impact on the expression levels of total STAT3, p65 and p38 in the rat aorta tissues. Treatment with DDE caused marked suppression of atherosclerosis-induced increases in levels of p-STAT3, p-p65 and p-p38, when compared to the model group. The atherosclerosis-induced elevations in p-STAT3,

p-p65 and p-p38 were suppressed to minimum levels in rats treated with DDE at a dose of 10 mg/kg.

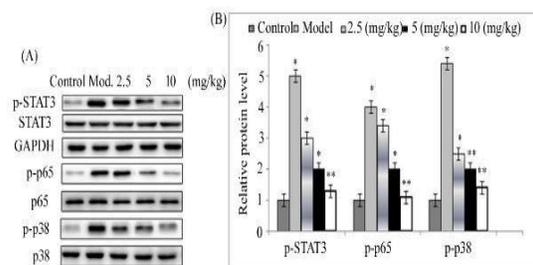


Figure 4: Effect of DDE on expressions of inflammatory pathway-associated proteins. (A) Changes in expression levels of p-STAT3, p-p65 and p-p38 in the aorta tissues of atherosclerotic rats, as determined using Western blot assay. (B) Quantified protein bands, with GAPDH as standard. * $P < 0.05$, ** $p < 0.02$, compared to sham group

DISCUSSION

Continuous administration of high-fat diet initiates a series of events in rat aortas which include elevated cytokine production, recruitment of immune cells and structural damage to the wall of aorta [14]. The histological features induced by these events in the aorta have close resemblance to those of atherosclerosis in humans. Chronic inflammation is associated with the pathogenesis of atherosclerosis. The initial stage of atherosclerosis is characterised by excess generation of pro-inflammatory cytokines in cardiovascular cells [15].

Endothelial as well as vascular muscle cells are cardiovascular cells that produce high levels of IL-1 β , IL-6, MCP-1 and TNF- α during chronic inflammation [15]. Several other cardiovascular cells are activated by these cytokines, leading to disturbance in homeostasis and activation of inflammatory response in the aorta wall. It has been reported that suppression of inflammatory response in animal models leads to inhibition of atherosclerosis [16]. In the present study, atherosclerosis led to a significant increase in average lesion area in the rat aortas. However, the average lesion area was significantly lowered in aortas of the atherosclerotic rats on treatment with DDE. This indicates that DDE inhibited atherosclerosis-induced aorta wall damage in rats.

The levels of IL-1 β , IL-6, MCP-1 and TNF- α in aorta tissues of the atherosclerotic rats were significantly increased, when compared to sham group. In DDE-treated rats, the atherosclerosis-induced elevations in levels of IL-1 β and IL-6

were significantly reversed, when compared to the model group. Treatment with DDE also reversed atherosclerosis-mediated upregulation of MCP-1 and TNF- α levels in rat aortas. These findings indicate that DDE alleviated the aorta damage due to atherosclerosis-induced inflammatory response in the rats.

It has been established that RAAS plays a key role in activation of vascular damage induced by inflammatory response which leads to formation of fatty streaks and foam cells, followed by rupture of the vulnerable plaques [17]. The renin-angiotensin-aldosterone system is also associated with maintenance of sodium balance and volume of the extracellular fluids. Many RAAS antagonists such as ACEIs and ARBs have been shown to inhibit inflammatory processes *in vivo* [18]. In the present study, atherosclerosis increased the levels of AngII, AT1 and AT2 in rat aortas. However, DDE treatment led to marked reversal of the atherosclerosis-induced increases in levels AngII, AT1 and AT2.

The NF- κ B pathway regulates several inflammatory responses, and is also associated with the progression of atherosclerosis [19]. The deactivation of NF- κ B via endothelium restriction has been demonstrated to suppress the formation of atherosclerotic plaques [20]. Moreover, it has been reported that the JAK/STAT pathway is involved in the pathogenesis of atherosclerosis [21]. Activated JAK/STAT is present in atherosclerotic lesions [22]. Incubation of vascular cells with AngII or cytokines has been shown to activate the JAK/STAT pathway [23]. In the present study, atherosclerosis significantly enhanced the expression of p-p65 in the aortas of the rats. Moreover, the expression levels of p-STAT3 and p-p38 were up-regulated in atherosclerotic rats, when compared to the sham group. However, treatment with DDE led to down-regulation of the expressions of p-STAT3, p-p65 and p-p38 in the aorta tissues of the atherosclerotic rats.

CONCLUSION

These results indicate that DDE protects rats from atherosclerosis-induced damage to aorta wall and inflammatory response. Moreover, RAAS expression, inflammatory cytokines and JAK/STAT signalling pathway are down-regulated in atherosclerotic rats on treatment with DDE. Thus, DDE is a potential remedy for the treatment of atherosclerosis in humans.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this manuscript and all liabilities pertaining to claims relating to the content of this manuscript will be borne by the authors. Huawei Tian and Yuping Li contributed to this work equally. Huawei Tian and Yuping Li performed the experimental work, carried out the literature survey, and analysed and compiled the data. Jun Zhang designed the study and wrote the manuscript. All the authors read the paper and approved it for publication.

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