

The protective effect of North Schisandra Lignans on vascular endothelial cell oxidation injuries

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Abstract.

BACKGROUND: In this study, the authors cultivated ECV-304 in vitro and incubated cells with H₂O₂, established injury models, and induced oxidized endothelial cell apoptosis. This model makes it possible to choose suitable concentrations of North Schisandra Lignans.

OBJECTIVE: To study the protective effects of North Schisandra Lignans on human umbilical vein endothelial cell injuries.

METHODS: Endothelial cell growth and proliferation activity were detected through the MTT method. The colorimetric method was used to determine superoxide dismutase (SOD) activity in the cell culture solution, as well as malondialdehyde (MDA) content in the cell.

RESULTS: North Schisandra Lignans noticeably decreased ECV-304 cell injury induced by H₂O₂. Moderate and high concentrations of North Schisandra Lignans could significantly lower MDA content and heighten SOD activity. These differences were significant compared to the H₂O₂ group ($P < 0.05$).

CONCLUSIONS: North Schisandra Lignans had an obvious protective effect on ECV-304 injured by H₂O₂. The mechanism decreases MDA production and heightened SOD activity.

Keywords: North Schisandra Lignans, vascular endothelial cell, superoxide dismutase, malondialdehyde

1. Introduction

Atherosclerosis (As) is a common cardiovascular disease that relates to vascular endothelial cell injury (VEC) [1,2]. The feature of As is the pathological change of involved arterial is usually from tunica intima and combined with multiple changes such as local accumulation of lipid and complex carbohydrate, fibrous tissue hyperplasia, calcium deposit, artery medial lysis, the secondary pathological changes also includes plaque entorrhagia, rupture and local thrombopoiesis [3]. Other arteriosclerosis pathological

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changes revealed by modern Cellular and Molecular Biology include macrophages vagile, smooth muscle cellular proliferation, quantity collagen fibers, elastic fibers and proteoglycans form into connective tissue matrix, lipid accumulates in or out cells [4,5]. Vascular endothelial cellular oxidative damage is considered to be an initiating agent of As.

Endothelial dysfunction often plays an important role in diseases such as As [9] and hypertension [6]. As the main accommodator of hemal dynamic balance, vascular endothelial cells can inhibit vascular smooth muscle cellular growth by expanding blood vessels and inhibiting inflammatory reactions to protect blood vessels. These effects are mainly mediated by NO, which is the most efficient endogenous vasodilating agent synthesized by NOS. NO can inhibit low density lipoprotein oxidation [7]. Evidence has shown that endothelial dysfunction, which can be detected by opacification or ultrasonic testing before vessel wall structure change occurs [8], is the prophase marker of As. Along with the deeply study on the mechanism of vascular endothelium injury, oxidative stress is more and more concerned. Oxygen radicals mediate oxidative stress, called active oxide (ROS). ROS-caused oxidative stress can lead to As. Besides oxidative stress, the signal passageway mediated by active oxygen may relate to monocytic adhesion and imbibition, thrombocytic activation, and contractile fiber cellular migration, which occurs in As [9,10].

Studies have shown that North Schisandra Lignans has obvious anti-oxidant effects. According to recent empirical studies and clinical trials, North Schisandra Lignans separated from *Schisandra chinensis* prevents atherogenesis by inhibiting oxidative low density lipoprotein, endothelial cells and monocyte adhesion, contractile fiber cellular immigration and proliferation, and macrophages cholesterol accumulation. It also promotes inflammatory cytokines and platelet aggregation expression [17,18] to protect the heart [11]. North Schisandra Lignans also have anti-oxidative stress protective effects on diabetics [12].

2. Material

2.1. Cell strain

HUVEC (ECV-304) was supplied by Jiang Su University.

2.2. Reagents

North Schisandra Lignans were supplied by Shanghai Vinhaket Biotechnology Co., Ltd, at 4–5°C iced storage. DMEM, EDTA, Trypsin, DMSO, MTT, acrylamide, glycine, and SDS were from American Sigma Co., LTD. Newborn calf serum, SOD kit, MDA kit, methylene bisacrylamide, and Tris were from American Amresco Co., LTD. Ammonium persulfate, TEMED, and PVDF were from American Promega Co., LTD. iNOS-actin antibody, IgG antibody marked by peroxidase, and NO kit were from American SantaCruz Co., LTD.

2.3. Instruments

Gel Doc XR gel imagery analytical system (Bio-Rad, American); SG ultrapure water machine (Germany); ELISA (RT-2100C, American); –70°C ultra cold freezer (HOF-382, Japan); Olympus inverted phase contrast microscope (IX70, Japan); CO₂ incubator (Shel-lab Co., LTD.); Western blot transmembrane machine (Bio-Rad, American).

3. Methods

3.1. Cell culture and grouping

ECV-304 cells were cultured in 15% fetal calf serum DMEM, 37°C, 5% CO₂, and 0.25% trypsinase digestive. There was one generation every 2d. Exponential phase cells were used in the next experiment. The cells were divided into four groups: control group, discard pro-media, add 10% fetal calf serum DMEM, culture for 24 h; H₂O₂ group, add 1 mmol/L H₂O₂ into 10% fetal calf serum DMEM; three different concentration drug groups, 30 min before adding 1 mmol /L H₂O₂, add 15 mg/L, 30 mg/L, 60 mg/L North Schisandra Lignans, further culture for 24 h. Cells and supernatant fluids were collected; the morphologic changes of every group were observed under an inverted phase contrast microscope. The MTT method was used to test cellular activity. We then detected the MDA, SOD, and NO content of each group's cellular supernatant fluid. Set six double-pored each group. Each experiment was repeated three times.

3.2. The MTT colorimetric method to assess cell viability

Exponential phase ECV-304 cells (1×10^8 /L), vaccinate on 96-wells cell culture plate, add 100 μ L unicell suspension each hole, culture for 24 h in CO₂ incubator, deal with the cells after adherence, 6 ~ 8 double-pored each group. 24 h later, 18 μ L (5 g/L) of MTT was added into each hole; they were incubated for 4 h at 37°C. Supernatant fluid was softly sucked away and discarded. 150 μ L of DMSO was added to each hole; they were agitated for 15 min to thoroughly dissolve the blue crystals. A 490 nm wave length was used to determine the OD. The cell multiplication inhibition ratio was calculated according to the OD. The Inhibition ratio(%) = $(1 - \text{drug group OD}/116001\text{control group OD}) \times 100\%$.

3.3. MDA content detection

The penthiobarbital acid coloration method was applied. The procedure was completed according to the MDA kit's instruction manual. 100 μ L cellular culture solution, 532 nm detect the absorbance, calculate MDA content according to formula. MDA content (μ mol/L) = $(\text{determined tube absorbance} - \text{determined blank tube absorbance})/(\text{standard tube absorbance} - \text{standard blank tube absorbance}) \times \text{proof sample concentration} + \text{dilution multiple of sample before detection}$.

3.4. SOD content detection

The Xanthine oxidase method was applied. The procedure was conducted according to the SOD kit's instruction manual. 100 μ L cellular culture solution, 550 nm detect the absorbance, calculate SOD content according to formula. SOD content (μ mol/L) = $(\text{control tube absorbance} - \text{determined tube absorbance})/\text{control tube absorbance}/50\% \text{ dilution multiple of reaction system} \times \text{dilution multiple of sample before detection}$.

3.5. NO content detection

The Nitrate reductase method was applied. The procedure was conducted according to the NO kit's instruction manual. 1×10^5 vaccinated cells per hole were placed into 24 wells on a culture plate (4 wells per group), grouping and dealing like above. Ending culture later, collect each group's supernatant fluid 100 μ L, 550 nm detect the absorbance, calculate NO secretory.

Table 1
Effect of North Schisandra Lignans on ECV-304 cellular activity. ($n = 3, \bar{x} \pm s$)

Groups	Dose	OD	Inhibition ratio (%)
Control group	–	0.689 ± 0.043	–
H ₂ O ₂ group	–	0.314 ± 0.025**	55.5211 ± 4.126
North Schisandra Lignans group	15 mg/L	0.413 ± 0.045	32.589 ± 2.541
	30 mg/L	0.569 ± 0.065 [#]	17.354 ± 1.365 [#]
	60 mg/L	0.702 ± 0.072 ^{##}	8.563 ± 0.896 ^{##}

**Compared with control group, $P < 0.01$; [#]Compared with H₂O₂ group, $P < 0.05$. ^{##}Compared with H₂O₂ group, $P < 0.01$.

3.6. Western blot

Cell groups were collected, protein was extracted, and protein concentration was detected via the Bradford method. 8% polyacrylamide gel was used and 30 µg of protein was added per swimming lane; the electrophoresis pressure was 120 V for 90 min. A damp-dry transfer method was used to transfer the protein in the separation gel to the PVDF membranes. 5% evaporated skimmed milk TBS-T buffer (25 mM Tris, 150 mM NaCl, 1% Tween 20, pH7.5), shaking 1 h, blocking non-specificity sites on PVDF membrane. The PVDF membrane was washed with a Tris buffer 3 times 10 min each time. Ab was added (1:1000 diluted) and kept at 4°C overnight. On the second day, a second Ab dilution (1:2000 diluted) and membrane were incubated at room temperature for 1 h and washed 3 times with TBS-T on a swing bed, 10 min each time, do chemoluminescence reaction, β -actin as internal reference. The results were collected with a gelatin imaging system and grayscale value detection was performed. The ratio between the sample's protein strap grayscale value and the β -actin was considered to be relevant for protein expression relative amount.

4. Statistics

The data was indicated as $\bar{x} \pm s$. SPSS 13.0 software was used to analyze the results. The variance analysis proceeded according to One Way-ANOVA. Comparison apply Student' T test, $P < 0.05$ stands for significant difference.

5. Results

5.1. The effect of North Schisandra Lignans on ECV-304 cellular activity

Treated by H₂O₂, vascular endothelial cellular activity decreased noticeably compared with the control group; the difference was significant ($P < 0.01$). The cellular fractional inhibition rate went up to 54%. Adding 15 mg/L, 30 mg/L, or 60 mg/L North Schisandra Lignans in advance improved vascular endothelial cellular activity and decreased the H₂O₂ treated cellular inhibition ratio. Moderate and high North Schisandra Lignans concentration + H₂O₂ displayed significant differences compared with the H₂O₂ group ($P < 0.05$, $P < 0.01$); the cellular activity of the high concentration drug group approached that of the control group, as shown in Table 1.

5.2. Effect of North Schisandra Lignans on ECV-304 cellular supernatant fluid MDA and SOD content

24 h after being treated by H₂O₂, ECV-304 cellular supernatant fluid MDA content increased ob-

Table 2
Effect of North Schisandra Lignans on ECV-304 cellular supernatant fluid MDA and SOD content. ($n = 3$, $\bar{x} \pm s$)

Groups	Dose	MDA/(μ mol/L)	SOD/(IU/mL)
Control group	–	1.106 \pm 0.131	18.782 \pm 2.101
H ₂ O ₂ group	–	2.327 \pm 0.497**	7.079 \pm 1.646**
North Schisandra Lignans group	15 mg/L	2.011 \pm 0.172	10.325 \pm 1.736
	30 mg/L	1.853 \pm 0.158##	12.574 \pm 1.852#
	60 mg/L	1.546 \pm 0.144##	15.441 \pm 1.931##

**Compared with control group, $P < 0.01$; #Compared with H₂O₂ group, $P < 0.05$. ##Compared with H₂O₂ group, $P < 0.01$.

Table 3
Effect of North Schisandra Lignans on ECV-304 cellular supernatant fluid NO content. ($n = 3$, $\bar{x} \pm s$)

Groups	Dose	NO/(μ mol/L)
Control group	–	69.231 \pm 4.685
H ₂ O ₂ group	–	54.365 \pm 1.328**
North Schisandra Lignans group	15 mg/L	69.589 \pm 2.135
	30 mg/L	75.354 \pm 2.987#
	60 mg/L	81.236 \pm 3.245##

**Compared with control group, $P < 0.01$; #Compared with H₂O₂ group, $P < 0.05$; ##Compared with H₂O₂ group, $P < 0.01$.

viously, but SOD content decreased obviously compared with the control group; the difference was significant ($P < 0.01$). Adding 15 mg/L, 30 mg/L, or 60 mg/L North Schisandra Lignans in advance improved SOD content and decreased MDA content. Moderate and high North Schisandra Lignans concentration + H₂O₂ displayed a significant difference compared with the H₂O₂ group ($P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.01$), as shown in Table 2.

5.3. Effect of North Schisandra Lignans on ECV-304 cellular supernatant fluid NO content

24 h after being treated by H₂O₂ ECV-304 cellular supernatant fluid NO content decreased significantly compared with the control group; the difference was significant ($P < 0.01$). Adding 15 mg/L, 30 mg/L, or 60 mg/L North Schisandra Lignans in advance improved NO content. Moderate and high North Schisandra Lignans concentrations + H₂O₂ displayed significant difference compared with the H₂O₂ group ($P < 0.05$, $P < 0.01$), as shown in Table 3.

5.4. Effect of North Schisandra Lignans on ECV-304 cellular eNOS expression

The result of the Western blot showed a specific strap at the molecular weight 133 KD site. 24 h after being treated by H₂O₂, ECV-304 cellular eNOS expression decreased significantly compared with the control group; the difference was significant ($P < 0.01$). Adding 15 mg/L, 30 mg/L, or 60 mg/L of North Schisandra Lignans in advance improved cellular eNOS expression. Moderate and high North Schisandra Lignans concentration + H₂O₂ displayed significant differences compared with the H₂O₂ group ($P < 0.05$, $P < 0.01$), as shown in Fig. 1.

6. Summary

Atherosclerosis is the pathologic cause of most cardiovascular diseases. Endothelial cell dysfunction is the first step during atherogenesis [13,14]. This leads to endothelial cell dysfunction, further increasing

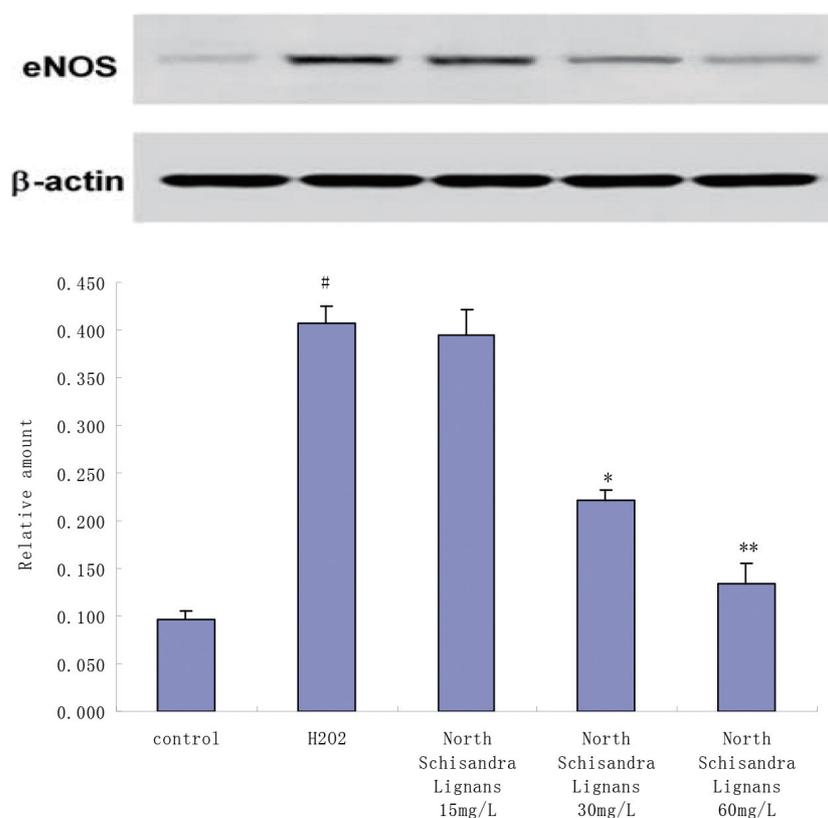


Fig. 1. Effect of North Schisandra Lignans on ECV-304 cellular eNOS expression. *Compared with control group, $P < 0.05$; **Compared with control group, $P < 0.01$; #Compared with H_2O_2 group, $P < 0.05$.

risk associated with cardiovascular diseases [15]. Many factors can induce endothelial cell dysfunction; oxidative stress plays an important role in cardiovascular disease pathogenesis because it can injure endothelium, inhibit NO production, increase active oxygen (ROS) production, and promote vascular endothelial dysfunction [16]. Currently, there is not a reliable method to detect endothelial dysfunction. H_2O_2 is considered a chief component of endothelial dysfunction. Vascular endothelial cellular activity can reflect cellular metabolism and proliferation. Recently, it was proved that the MTT method can be used to study endothelial cellular activity. MTT can be reduced into insolubility amethyst crystallization by succinate dehydrogenase of viable cellular mitochondria and deposit it in cells. However, dead cells do not have this function; thus, this method consensual reflect cytoactive. At a certain cell number, MTT crystallization is a direct ratio to the cell number. Our MTT result shows that North Schisandra Lignans can improve the cytoactivity of ECV-304 treated by H_2O_2 . It proved that North Schisandra Lignans can promote hydroxy radical production by oxidizing biomacromolecules such as lipid to foment vascular endothelial cellular injury.

H_2O_2 can mediate cellular oxidative stress and increase intra-cellular ROS, which attacks various biomacromolecules such as DNA, RNA, protein, fat etc., then causes the alteration of cellular structure and function. Therefore, detecting MDA amounts can show the degree of lipid peroxidation, and further indirectly reflect the degree of cellular injury. Superoxide dismutase (SOD) can reveal the body's anti-oxidative stress system cleaning ROS ability. Our results show that North Schisandra Lignans can

improve SOD content and decrease lipid peroxidation. This suggests that North Schisandra Lignans has obvious antioxidant capacity.

As oxidative stress plays an important role in atherosclerotic development. Oxidative stress injure vascular endothelium inhibits NO production, which is a kind of endogenous vasodilating factor with doughty vasodilation roles. NO can inhibit ROS production and has an obvious anti-oxidant stress effect. NO is produced through the L-arginine-NO-cGMP passageway, where nitric oxide synthetase (NOS) is the key enzyme. NOS includes cNOS and iNOS; the former also includes the endothelial cell type (eNOS) and cerebral type (nNOS). Being vasodilating, NO is mainly synthesized through eNOS. NO synthesized by eNOS is the most effective endogenous vasodilating agent, which has the roles of ri-valry endogenous amidephrine, and inhibits low density lipoprotein oxidation, platelet aggregation, and contractile fiber cellular multiplioation. Our results suggest that North Schisandra Lignans regulates NO production through eNOS.

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