Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

The clinical effects of resveratrol on atherosclerosis treatment and its effect on the expression of NADPH oxidase complex genes in vascular smooth muscle cell line

Guo Lixia[1], Zhao Haiyun[2], Zheng Xia[3*]

[1] Pharmacy Division, Huangdao District Chinese Medicine Hospital, Qingdao, China
[2] Dressing Room, Huangdao district Chinese Medicine Hospital, Qingdao, China
[3] Department of Cardiology, Huangdao district Chinese Medicine Hospital, Qingdao, China

*Correspondence to: zhengxiamjn@163.com

Received July 28, 2021; **Accepted** September 30, 2021; **Published** November 22, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.3.22

Abstract: Atherosclerosis is a disease that covers the arteries of the middle to large arteries and can affect the aorta to the coronary arteries. Atherosclerosis is a progressive and slow process that begins in childhood and leads to clinical manifestations in adulthood. Increased activity of the NADPH oxidase complex plays an important role in the development of this complication. In this study, the clinical effects of resveratrol were considered on atherosclerosis treatment and its effect on the expression of NADPH oxidase complex genes was performed in the vascular smooth muscle cell line. For this purpose, 120 Chinese Patients prone to atherosclerosis participated for 12 months. These patients were divided into two groups of 60 patients. The first group was treated to the placebo and the second group was treated to 100 mg/day resveratrol for 12 months. Also, the expression of $gp91^{Phox}$ and P^{22Phox} subunits of NADPH oxidase complex was evaluated in vascular smooth muscle cells by real-time PCR technique. The results showed that resveratrol could decrease systolic blood pressure, diastolic blood pressure, cholesterol, triglyceride, and low-density lipoprotein (LDL). It was also able to reduce the expression of $gp91^{Phox}$ and P^{22Phox} subunits of NADPH oxidase complex in the vascular smooth muscle cell line. Therefore, it is possible to introduce resveratrol as an able medicine to treat atherosclerosis. However, more studies are needed to complete the information about the role of resveratrol.

Key words: Atherosclerosis; NADPH oxidase complex; Resveratrol; VSMC line.

Introduction

Atherosclerosis is characterized by the deposition of lipids and other substances in the inner wall of some arteries. The result of this process is the formation of fibrous-fat plaques (atheroma), which increases with age and causes narrowing of the arteries (1). The disease is one of the leading causes of death in adults and imposes huge costs on communities. Atherosclerosis begins with the formation of fatty streaks in the intima layer of the arteries and eventually progresses to fibro stroma plaque, fibrous plaque, and complication plaques. Inflammatory activity has been observed in many immune cells present in atheroma, and many cytokines are produced by these cells (2). Atrophy of calcification according to an active and regulated process in the vessel wall starts from the beginning of atheroma formation and with increasing calcification, the probability of atrophy rupture increases. The cause of vein calcification is the differentiation of vascular cells following stimulation with cytokines, inflammatory factors, and altered lipoproteins in atrophic plaques (3). Atherosclerotic calcification is similar to calcification and bone formation in terms of osteoblast and chondrocyte differentiation, ossification, and bone matrix deposition (4).

Studies have shown that calcified arteries and vascular cells in the culture medium are exposed to oxidative stress (5-7). One of the main sources of oxygen-free radical production is the enzymatic complex of NADPH oxidase (8). The expression of this enzyme is well demonstrated in endothelial cells, fibroblasts, vascular smooth muscle cells, and cardiomyocytes (9). NOX is a catalytic enzyme of the NADPH oxidase, which has 5 different isoforms expressed in the body (10). NOX2 ($gp91^{Phox}$) has received a great deal of attention due to its significant expression and activity in the heart. The $gp91^{Phox}$ along with another subunit called P^{22Phox} form the membrane components of this complex (11). The function of this enzymatic complex is highly dependent on cytoplasmic subunits including P^{47Phox}, P^{67Phox}, and a small protein attached to GTP called Rac. Phosphorylation of P^{47Phox} causes a spatial change in the protein and its displacement to the NOX2/P^{22Phox} complex. As P^{47Phox} is localized in the membrane, it also carries the P^{67Phox} protein (subunit activator) and a smaller unit called P^{40Phox}. Finally, Rac1 connects to NOX2 and then to P^{67Phox} (12). As soon as the components are assembled, the system is activated and a superoxide anion is produced by transferring electrons from NADPH to oxygen. Studies have shown that NADPH oxidase is activated in response to stimuli such as angiotensin-II, endothelin-I, cytokines, and mechanical traction in heart cells. These are events that are quite evident in the process of atherosclerosis, so that along with calcification, this enzymatic complex also increases (13, 14).

Due to the high prevalence of atherosclerosis and

mortality of patients, the need to find more effective treatments is the goal of medical researchers. In recent years, the cardio-protective effects of phenol from plant origin called resveratrol (3,4,5-tri-hydroxy-trans-acetyl-ben-1acetyl benoid) have been considered (15). This medicine is from the class of flavonoids, which is chemically classified with the formula $C_{14}H_{12}O_3$ in a group of compounds called acetylben. This natural phenol is found in black grapes, berries, peanuts, and black tea. Researchers attribute this phenol to the activation of the enzyme deacetylase SIRT1 by its anti-inflammatory, antioxidant, and vasodilator effects (16).

In this study, in addition to the clinical effect of resveratrol on the treatment of atherosclerosis, its effect was also evaluated on the expression of $gp91^{Phox}$ and P^{22Phox} subunits of the NADPH complex in the vascular smooth muscle cell line.

Materials and Methods

Patients

In this study, 120 Chinese Patients prone to atherosclerosis participated for 12 months. All of these patients were treated to 20 mg/day atorvastatin (Pfizer, Germany). These patients were divided into two groups of 60 patients. The first group used atorvastatin and placebo. The second group, in addition to taking atorvastatin, received 100 mg/day resveratrol for 12 months. Demographic and clinical information of patients is given in Table 1.

Cell culture and treatment

In this experimental study, vascular smooth muscle cell (VSMCs) lines, which were prepared from Cell Bank Australia, were cultured in an F12K medium. This culture medium contains 0.05 mg/ml ascorbic acid, 0.01mg/ml insulin, 0.05mg/ml transferrin, 10ng/ml sodium selenite, 0.03mg/ml endothelial cell growth supplements (ECGS), 10% FBS, HEPES 10mM, 100U/ml penicillin, 100U/ml streptomycin and 0.01% amphotericin. To have an estrogenic medium, 10mM beta-glycerophosphate was added to the medium. The cells were cultured in a suitable flask in an incubator at 37°C and 5% CO_2 at suitable humidity. Daily control of cultured cells in terms of growth conditions and cell division, etc. was performed and passed as needed. After cell growth and proliferation, passages 4 to 6 were used for experiments. The cells were divided into plates of 12 culture houses at the rate of 15,000 per well. When the cell density reached 80%, the cells were treated with 10µg/ml 6-mercaptopurine to induce oxidative stress. Then half of them were treated with resveratrol prepared from Bio Vendor Company against the control group. The control group did not treat with 6-mercaptopurine and of resveratrol. The concentration of resveratrol used in these experiments was 5µg/ml. After 24 and 48 hours in the presence of 6-mercaptopurine and resveratrol cells, the treated cells and control cells (without resveratrol) were trypsinized and pooled to extract RNA at later stages.

RNA extraction and cDNA synthesis

RNA was extracted by bioflux kit (Bioflux-Malaysia) according to the relevant instructions and its concentration and quality were determined by small-scale spectrophotometry (Nano-drop). Three micrograms of each RNA sample were used to make the cDNA by Thermo-Canada kit.

Expression of $gp91^{Phox}$ and P^{22Phox} genes

Gene expression was assessed by Real-Time PCR using Syber green by Rotor-Gene (Corbett 3000-Australia). GAPDH gene was used as the reference gene and the expression of genes in the treated and control groups was measured. Three replications were performed for each sample. The sequence of primers used is given in Table 2. The test conditions were: 5 minutes of enzyme activation at 95°C, 40 cycles including 95°C for 15 seconds, 59°C ($gp91^{Phox}$) and 57°C (P^{22Phox}) for 20 seconds and 72°C for 30 seconds.

After the experiment, in order to confirm the amplification of specific fragments of each gene and the absence of nonspecific products and dimer primer, the Melting Curve diagram was examined for each gene. Reaction data were analyzed by the $2^{-\Delta\Delta ct}$ method.

Statistical Analysis

Table 1. Demographic and clinical information of patients in two groups.

Characteristic	First group	Second group	*P*-value
Mean age (year)	52.8±4.7	53.1±77.3	0.349
Systolic Blood Pressure (mm Hg)	141.8±9.3	144.1±10.1	0.286
Diastolic Blood pressure (mm Hg)	94.3±3.1	94.4±4.3	0.898
Smoking patient	18.1%	17.9%	0.464
BMI (Kg/m^2)	28±2	27±2	0.135
Diabetes II	13.6%	12.8%	0.105

Table 2. The sequence of primers and product length.

Gene		Primer Sequence	Product Length
GAPDH	Forward	3′-ACACCCACTCCTCCACCTTTG-5′	112bps
	Reverse	3′-TCCACCACCCTGTTGCTGTAG-5′	
$gp91^{Phox}$	Forward	5′-GAATGAGGGGCTCTCCATTT-3′	267bps
	Reverse	5′-CAACCTCTCACAGAGATACA-3′	
P^{22Phox}	Forward	5′-CAGCCGGGTTCGTGTCGC-3′	596bps
	Reverse	5′-TCGTCGGTCACCGGGATG-3′	

Table 3. Results of resveratrol treatment in two groups of placebo and treatment group, before and 12 months after starting treatment.

Variables	Placebo group		Treatment group	
	Start	12 months later	Start	12 months later
Systolic Blood Pressure (mm Hg)	141.8±9.3	140.8±8.7	144.1±10.1	130.7±8.2*
Diastolic Blood pressure (mm Hg)	94.3±3.1	95.3±6.2	94.4±4.3	87.19±3.1*
Cholesterol (mg/dl)	231.9±35.5	229.9±42.5	230.1±41.3	172.5±47.9**
Triglyceride (mg/dl)	290.9±31.3	291.9±41.9	295.1±40.2	162.5±44.7*
High-density lipoprotein (HDL) (mg/dl)	40.9±9.3	40.4±8.1	40.01±8.1	39.9±7.3
Low-density lipoprotein (LDL) (mg/dl)	150.1±27.8	131.2±33.6	147.8±33.6	90.2±36.3**
Aspartate Transaminase (AST) (U/l)	23.5±5.2	24.1±4.1	24.8±5.3	26.2±4.6*
Alanine Transaminase (ALT) (U/l)	25.7±3.2	26.2±4.1	26±4.6	26.9±3.1

* $P<0.05$; **$P<0.01$

Data analyses were performed with SPSS 16 software and Graph Pad Prism software, and they were analyzed by one-way ANOVA and Tukey test. $P<0.05$ was considered for significant differences between the experimental groups. The obtained values were reported as Mean ±standard error.

Results

Clinical results

Clinical results are listed in Table 3, before and after the experiment. The first group, who were treated with atorvastatin and placebo, did not have a significant change in their treatment, after 12 months. But the second group, which in addition to taking atorvastatin, they received resveratrol daily, showed a significant decrease in the amount of systolic blood pressure, diastolic blood pressure, cholesterol, triglyceride, and low-density lipoprotein (LDL), and a significant increase in aspartate transaminase. But no significant change was seen in high-density lipoprotein (HDL) and alanine transaminase levels in this group, after 12 months.

Gene expression results

To induce oxidative stress, 10µg/ml 6-mercaptopurine was used. The effect of 6-mercaptopurine was demonstrated on vascular smooth muscle cell (VSMC) lines in Figure 1. 6-mercaptopurine increased the expression of gene $gp91^{Phox}$ in VSMC lines 2.17 and 3.62 times during 24 and 48 hours, respectively. It also increased the expression of the P^{22Phox} gene 3.51 and 4.33 times.

In Figure 2, the effect of resveratrol was demonstrated on vascular smooth muscle cell (VSMC) lines treated with 6-mercaptopurine. About $gp91^{Phox}$, in 24 and 48 hours, the expression of this gene was increased in VSMC lines, in the presence of 6-mercaptopurine. But this expression was decreased in the presence of 6-mercaptopurine and resveratrol. The same results were obtained for the P^{22Phox} gene.

The diagram obtained from the melting curve showed the specific amplification of the target genes and the absence of primers pairing (Figure 3).

Discussion

According to the role of NADPH oxidase enzyme complex as an effective factor in the production of free radicals in atherosclerosis (14), and also the cardio-protective effects of resveratrol (17), in this study, we investigated the cardio-protective effects of resveratrol on the treatment of atherosclerosis and its effect on the expression of two NOX2 complex genes in vascular smooth muscle cell (VSMCs) lines.

The first part of the study showed that taking resveratrol at a dose of 100 mg per day for 12 months was able to significantly reduce systolic blood pressure, diastolic blood pressure, cholesterol, triglyceride, and low-density lipoprotein (LDL). The pathogenesis of atherosclerosis begins when the stores of LDL lipoprotein in the blood increase, and its accumulation and storage in the inner layer covering the arteries (Tunica Intima) begins, which is the first stage of the disease (18). LDL then diffuses through endothelial cell connections by diffusion

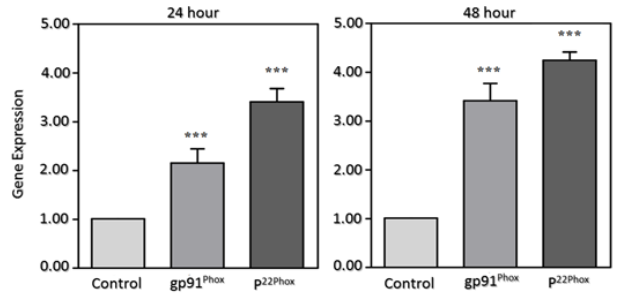

Figure 1. The effect of 6-mercaptopurine at a concentration of 10µg/ml on the expression of $gp91^{Phox}$ and P^{22Phox} genes in the VSMC line at 24 and 48 hours.

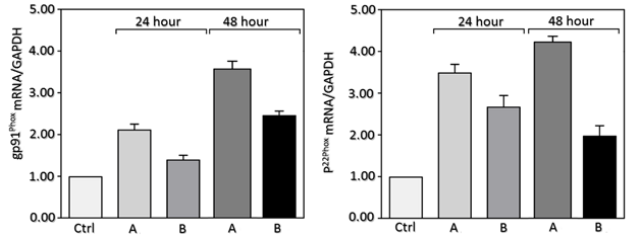

Figure 2. The effect of 5µg/ml resveratrol on the expression of $gp91^{Phox}$ and P^{22Phox} genes in the VSMC cell line at 24 and 48 hours; Ctrl: Control; A: VSMC lines treated with 6-mercaptopurine; B: VSMC lines treated with 6-mercaptopurine and resveratrol.

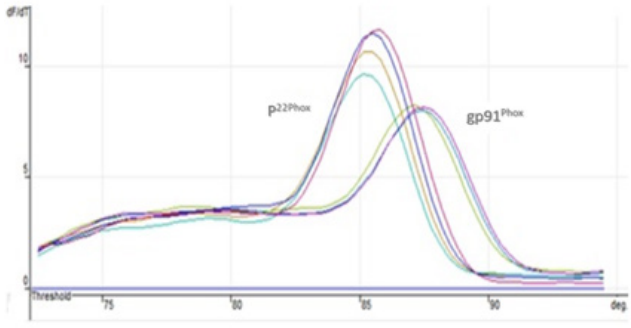

Figure 3. The real-time PCR reaction melting curve related to the expression of $gp91^{Phox}$ and P^{22Phox} genes.

or by its carriers and is deposited in the extracellular matrix proteoglycan (ECM) or reaches the middle layer (Tunica Media) of the artery wall from the inner elastic blade. Accumulation of LDL stimulates the adhesion of monocytes to the endothelium and ultimately facilitates their differentiation into macrophages (19). Macrophages are then activated to digest fats, a process that leads to the production of foam cells (FCs) by phagocytosis of lipids. Local accumulation of these foam cells leads to the production of fat streaks in the intima of the arteries (coronary arteries). The development of atherosclerosis continues with the involvement of T lymphocytes (20). In atherosclerosis, macrophages lead to the oxidation of LDL, which in turn leads to the development of FCs, leading to plaque development. With more accumulation of lipoproteins in macrophages and the addition of necrosis process, the central lipid core is formed and as this process continues, vascular smooth muscle cells migrate from the middle layer of the arteries (Tunica Media) and connective tissue consisting of Collagen fibers, elastic and proteoglycan fibers, and finally, a fibrous capsule is formed around the plaque (21). In the early stages of plaque growth, it increases the diameter of the vessel to the outside, but as plaque grows, it grows inside the vessel and decreases the inner diameter of the vessel (lumen) (18). In most cases, plaque complication, which includes plaque rupture, bleeding, and clotting, leads to acute coronary events. Plaque instability can depend on the amount of fat and connective tissue, inflammatory factors, coronary artery contraction, platelet activation, and coagulation (20). Therefore, resveratrol with decreasing the number of important factors, such as LDL, could prevent or treat atherosclerosis. On the other hand, resveratrol increased aspartate transaminase, in the patients group treated with resveratrol, that it showed resveratrol could have adverse effect on liver. Although this increase in enzyme levels was still within the standard range and safe for the liver, more studies are needed.

In the second part of the study, induction of oxidative stress was performed on vascular smooth muscle cell (VSMC) lines to study the changes in the expression level of NADPH oxidase enzyme and its components in atherosclerosis and also the effect of resveratrol on the expression of subunits of this complex on VSMC lines. Regarding the role of the NADPH oxidase enzyme in atherosclerosis, it should be mentioned that, in atherosclerosis, lipoproteins are oxidized. Oxidation of these lipoproteins by free radicals produced by macrophages and their enzymatic change in the intima causes the production of lipids that are vitally active (22). Endothelial and vascular smooth muscle cells in the atheroma environment may also play an important role in this process. These oxidized lipoproteins release phospholipids that activate endothelial cells, especially in areas of the vessel that are under hemodynamic pressure (23). Oxidized LDLs themselves stimulate inflammatory reactions, promoting the uptake of blood monocytes and the mobilization and proliferation of macrophages produced by the uptake of monocytes. These inflammatory reactions arise to eliminate oxidized LDLs (20). In this process, the oxidized fats activate the macrophages and intensify their oxidation phenomenon. In the presence of hypercholesterolemia, the inflammatory responses that are initiated to counteract the effects of oxidized LDLs cannot complete their function; instead, the inflammatory cycle, oxidation of lipoproteins, and further inflammation remain in the intima (23). This oxidation is caused by the NADPH oxidase enzyme and its sub-units (14). The results of the current study showed that resveratrol was able to greatly reduce the expression of $gp91^{Phox}$ and P^{22Phox} genes. These two genes are subunits of the NADPH oxidase complex. Therefore, this drug could reduce the production of ROS in VSMC lines with oxidative stress.

The results of our study showed that taking resveratrol at a dose of 100 mg/day for 12 months can prevent or treat atherosclerosis by decreasing systolic blood pressure, diastolic blood pressure, cholesterol, triglyceride, and low-density lipoprotein (LDL). Resveratrol was also able to reduce the expression of $gp91^{Phox}$ and P^{22Phox} subunits of NADPH oxidase complex in the vascular smooth muscle cell line. Therefore, some parts of the resveratrol cardioprotective effects may have occurred in this way.

References

1. Libby P. The changing landscape of atherosclerosis. Nature 2021; 592(7855): 524-533.
2. Libby P. Inflammation in atherosclerosis—No longer a theory. Clin Chem 2021; 67(1): 131-142.
3. van Leent MM, Beldman TJ, Toner YC et al. Prosaposin mediates inflammation in atherosclerosis. Sci Transl Med 2021; 13(584).
4. Gencer S, Evans BR, van der Vorst EP, Döring Y, Weber C. Inflammatory chemokines in atherosclerosis. Cells 2021; 10(2): 226.
5. Yang X, Chen A, Liang Q et al. Up-regulation of heme oxygenase-1 by celastrol alleviates oxidative stress and vascular calcification in chronic kidney disease. Free Radic Biol Med 2021; 172: 530-540.
6. Bilal I, Xie S, Elburki M, Aziziaram Z, Ahmed S, Jalal Balaky S. Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines. Cell Mol Biomed Rep 2021; 1(1): 14-22.
7. McCarty MF, Lerner A, DiNicolantonio JJ, Iloki-Assanga SB. High Intakes of Bioavailable Phosphate May Promote Systemic Oxidative Stress and Vascular Calcification by Boosting Mitochondrial Membrane Potential—Is Good Magnesium Status an Antidote? Cells 2021; 10(7): 1744.
8. Xiong H, Hua L, Reyna-Llorens I et al. Photosynthesis-independent production of reactive oxygen species in the rice bundle sheath during high light is mediated by NADPH oxidase. Proc Natl Acad Sci 2021; 118(25).
9. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: mechanisms of NADPH oxidase activation and bacterial resistance. Front Cell Infect Microbiol 2017; 7: 373.
10. Lambeth JD, Kawahara T, Diebold B. Regulation of Nox and Duox enzymatic activity and expression. Free Radic Biol Med 2007; 43(3): 319-331.
11. Zhang M, Kho AL, Anilkumar N et al. Glycated proteins stimulate reactive oxygen species production in cardiac myocytes: involvement of Nox2 (gp91phox)-containing NADPH oxidase. Circulation 2006; 113(9): 1235-1243.
12. Chen F, Yang D, Cheng X-Y et al. Astragaloside IV ameliorates cognitive impairment and neuroinflammation in an oligomeric Aβ induced Alzheimer's disease mouse model via inhibition of microglial activation and NADPH oxidase expression. Biol Pharm Bull 2021: b21-00381.

13. Fathi A., Barak M, Damandan M, Amani F, Moradpour R, Khalilova I., Valizadeh M. Neonatal Screening for Glucose-6-phosphate dehydrogenase Deficiency in Ardabil Province, Iran, 2018-2019. Cell Mol Biomed Rep 2021; 1(1): 1-6.
14. Xue S, Tang H, Zhao G et al. C–C motif ligand 8 promotes atherosclerosis via NADPH oxidase 2/reactive oxygen species-induced endothelial permeability increase. Free Radic Biol Med 2021; 167: 181-192.
15. Meng T, Xiao D, Muhammed A, Deng J, Chen L, He J. Anti-inflammatory action and mechanisms of resveratrol. Molecules 2021; 26(1): 229.
16. Amiri A, Mahjoubin-Tehran M, Asemi Z et al. Role of resveratrol in modulating microRNAs in human diseases: From cancer to inflammatory disorder. Curr Med Chem 2021; 28(2): 360-376.
17. Bostancıeri N, Elbe H, Eşrefoğlu M, Vardı N. Cardioprotective potential of melatonin, quercetin and resveratrol in an experimental model of diabetes. Biotech Histochem 2021: 1-6.
18. Mohindra R, Agrawal DK, Thankam FG. Altered vascular extracellular matrix in the pathogenesis of atherosclerosis. J Cardiovasc Transl Res 2021: 1-14.
19. Xu J, Kitada M, Ogura Y, Koya D. Relationship between autophagy and metabolic syndrome characteristics in the pathogenesis of atherosclerosis. Front Cell Dev Biol 2021; 9.
20. Poznyak AV, Bharadwaj D, Prasad G, Grechko AV, Sazonova MA, Orekhov AN. Renin-Angiotensin System in Pathogenesis of Atherosclerosis and Treatment of CVD. Int J Mol Sci 2021; 22(13): 6702.
21. Shen D, Wu Y, Zhao L. Roles of periodontal pathogens in the pathogenesis of atherosclerosis. Zhonghua Kou Qiang Yi Xue Za Zhi 2021; 56(6): 584-590.
22. TANG B-Y, WEN J, TANG X-H. Research progress on the role of ADAM17 in the pathogenesis of atherosclerosis. basic Clin Med 2021; 41(5): 744.
23. Lien C-F, Chen S-J, Tsai M-C, Lin C-S. Potential role of protein kinase C in the pathophysiology of diabetes-associated atherosclerosis. Front Pharmacol 2021; 12: 1674.