Microbes and Infection 25 (2023) 105177

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Protective effects of tanshinone IIA on *Porphyromonas gingivalis*-induced atherosclerosis via the downregulation of the NOX2/NOX4-ROS mediation of NF-κB signaling pathway

Yan Xuan [a], Cai Yu [b], Kang Ni [b], Lou Congcong [b], Qiu Lixin [a, *], Luan Qingxian [b, **]

[a] *Department of the Fourth Division, Peking University, School and Hospital of Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Laboratory for Digital and Material Technology of Stomatology & Beijing Key Laboratory of Digital Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, PR China*

[b] *Department of Periodontology, Peking University, School and Hospital of Stomatology & National Clinical Research Center for Oral Diseases & National Engineering Laboratory for Digital and Material Technology of Stomatology & Beijing Key Laboratory of Digital Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, PR China*

ARTICLE INFO

Article history:
Received 5 March 2023
Accepted 25 June 2023
Available online 29 June 2023

Keywords:
Porphyromonas gingivalis
Atherosclerosis
Tanshinone IIA
NOX2
NOX4
NF-κB

ABSTRACT

Tanshinone IIA (TSA), an active component isolated from Danshen, possess high medicinal values against atherosclerosis by reducing vascular oxidative stress, inhibiting platelet aggregation, and protecting the endothelium from damage. The periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) has been proven to accelerate the development of atherosclerosis. We aim to determine the effects of TSA on *P. gingivalis*-induced atherosclerosis in ApoE-knockout (ApoE−/−) mice. After feeding with a high-lipid diet and infected with *P. gingivalis* three times per week for four weeks, TSA-treated (60 mg/kg/d) mice greatly inhibited atherosclerotic lesions both morphologically and biochemically and exhibited significantly reduction ROS, 8-OHdG, and ox-LDL levels in serum compared with *P. gingivalis*-infected mice. Additionally, TSA-treated mice were observed a marked reduction of ROS, 8-OHdG and ox-LDL in the serum, mRNA levels of COX-2, LOX-1, NOX2 and NOX4 in the aorta, as well as the levels of NOX2, NOX4, and NF-κB. These results suggest that TSA attenuates oxidative stress by decreasing NOX2 and NOX4 and downregulating NF-κB signaling pathway, which might be contributed to the amelioration of atherosclerosis.

Atherosclerosis (AS) is a multifactorial cardiovascular disease and one of the most prevalent fatal conditions in the world [1]. Oxidative stress is considered to play a pivotal role in the development of AS and the formation of plaques [2]. *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative, non-motile, anaerobic bacterium, is not only one of the most important pathogens associated with chronic periodontitis but also a significant risk factor for AS [3]. Previous studies have shown that oral bacteria can enter the blood circulation system and reach distant organs, which has also been confirmed by our previous studies .

Previous studies have demonstrated the access of these bacteria to the systemic circulation and their existence in distant organs; these findings were confirmed in our previous study [4]. Several researchers have focused on the mechanisms of *P. gingivalis*-accelerated AS and highlighted the presence of oxidative stress, inflammation, and immune responses caused by these bacteria during the development of this disease [5,6]. Nonetheless, despite these clinical data analyses and scientific reports, the mechanism involved in the pathogenesis of *P. gingivalis*-accelerated AS remains unclear.

During the early stages of AS, oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds their clearance by antioxidant defenses. ROS are thought to play an important role in host defense against bacteria-induced tissue destruction. ROS are a group of unstable and highly reactive molecules and free radicals typically generated as by-products of cellular processes that involve oxidative stress [7]. Some studies have shown that the generation of excessive ROS results in the initiation and progression of cardiovascular diseases and represents a common and potent mediator of pathogenic risk factors associated with cardiovascular dysfunction [8]. The most important enzymes that produce ROS include nicotinamide-adenine dinucleotide phosphate (NADPH)

* Corresponding author.
** Corresponding author.
E-mail addresses: kqxuanyan@163.com (Q. Lixin), kqluanqx@126.com (L. Qingxian).

https://doi.org/10.1016/j.micinf.2023.105177

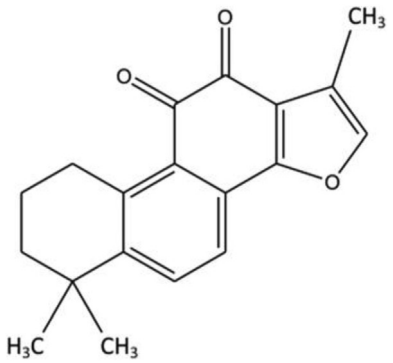

Fig. 1. Structure of tanshinone IIA (TSA).

oxidases (NOX enzymes), xanthine oxidase, the mitochondrial electron transport system, and NO synthase. NOX is responsible for producing O_2- and/or H_2O_2 by catalyzing the transfer of electrons from NADPH to molecular oxygen, which distinguishes them from other oxidase enzymes that produce ROS as a by-product. NOX2 and NOX4 are predominately expressed in myocardial cells [9]. NOX-derived ROS have been associated with fibrosis and are thought to regulate multiple inflammatory signal transduction pathways involved in the pathogenesis of cardiac dysfunction and myocardial infarction [10]. One study demonstrated the potential role of Tanshinone IIA (TSA, Fig. 1) against LPS-induced cardiac fibrosis in mice via inhibition of NOX2 [11]. Therefore, preventing vascular oxidative stress and inhibiting an overload of NOX may effectively treat atherosclerotic disease.

Tanshinone IIA (TSA) is a major bioactive lipophilic constituent isolated from Danshen, the root of the herb *Salvia miltiorrhiza* Bunge used in traditional Chinese medicine. TSA was found to have cardioprotective and anti-atherosclerotic effects due to its anti-oxidative, anti-inflammatory, and anti-adipogenic effects [12]. Therefore, the aim of this study was to determine whether the regulative use of TSA can prove effective against the development of *P. gingivalis*-induced AS and to investigate the molecular pathological mechanisms involved in this process.

1. Materials and methods

1.1. Animals

This study was approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center (approval number LA2020214). A total of 24 seven-week-old male ApoE-knockout (ApoE−/−) mice (C57BL/6) were obtained from Vital River Inc. (Beijing, China) and were adapted to their environment for 7 days before the experiment. The animals were housed under specific pathogen-free conditions at the Laboratory Animal Center of Peking University and provided with food and water *ad libitum*.

1.2. Bacteria

The *P. gingivalis* strain FDC381 was cultured in a humidified environment containing 80% N_2, 10% CO_2, and 10% H_2 and grown in Brain Heart Infusion broth (Oxoid Ltd., England) with 5% sheep blood anaerobic basal agar plates (Oxoid Ltd.) at 37 °C for 3–5 days until they reached an optical density (OD) of 1.0 at 600 nm (OD600), which corresponded to a colony forming unit (CFU) of 10^8/100 μL. Adherent rough-colony variants of bacteria were scraped into 5 mL of *P. gingivalis* growth medium and pelleted by centrifugation at 8000 *g* and 4 °C for 10 min; the pellet was resuspended with phosphate-buffered saline (PBS) for intravenous injection.

1.3. Allocation of the mice into groups

The mice were randomly divided into three groups based on the exposure to the various agents as follows: (a) treated with ApoE −/− + PBS (100 μL per mouse); (b) treated with ApoE −/− + *P. gingivalis*; and (c) treated with ApoE −/− + *P. gingivalis* + TSA (60 mg/kg/d). The mice in groups (b) and (c) received intravenous injections of *P. gingivalis* (10^8 CFU 100 μL^{-1} per mouse) three times per week for 4 weeks. TSA (Sigma–Aldrich, USA) was dissolved in 0.5% carboxymethylcellulose (CMC; Sigma–Aldrich, USA) for oral gavage at a dose of 60 mg/kg/day in group (c) [13].

1.4. Tissue collection

The mice received a high-fat diet and distilled water with (c) or without (a, b) TSA for 4 weeks before they were euthanized by pentobarbital sodium (intraperitoneal injection; 100 μg μL^{-1}; Merck, Germany). Blood samples were collected by infraorbital puncture, and serum was isolated by centrifugation at 10,000 rpm and 4 °C for 5 min; the serum was stored at −80 °C for enzyme-linked immunosorbent assay (ELISA). The heart and aorta were perfused with heparinized ice-cold 0.9% PBS through the left ventricle for 10 min. Subsequently, they were excised and washed in icy normal saline. Half of the heart and aorta were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA, USA) to prepare the frozen sections. The other halves were used for Western blot analysis and quantitative real-time polymerase chain reaction (qRT-PCR).

1.5. Histopathological analysis

Cryosections of the heart (including the aortic root) were carefully dissected, and embedded in OCT, and which were stained for with Oil Red O staining. The number of atherosclerotic lesions were counted following according to our previous study [4]. Each section of the aortic sinus (thickness, 5 μm thickness) was captured by via optical microscopy (Nikon Eclipse Ci, Japan), performed using an Olympus Q Color 5 digital camera and the number of atherosclerotic lesions were calculated using the digital Pro-Plus Software 6.0 (Media Cybernetics, USA). The values obtained from 15 sections per animal were averaged and expressed as the percentage of the lumen in the proximal aorta that was occupied by the lesions per section per animal.

1.6. ELISA

Serum samples from the mice in the three groups were separated from blood after euthanasia. The concentration of total protein was determined using the protein assay reagent (RayBiotech, USA, Inc., USA), following which the total protein was incubated with ROS, 8-hydroxylated deoxyguanosine (8-OHdG) and oxidized low-density lipoprotein (ox-LDL) ELISA kits (RayBiotech, USA) at 37 °C for 1 h. The absorbance was measured at 450 nm using an ELISA analyzer (DNM-9606; Perlong Medical, Jiangsu, China).

1.7. qRT-PCR

Total RNA was extracted from the aorta using the RNeasy Fibrous Tissue Kit (Qiagen, Germany) and converted into cDNA Primescript RT Master Mix Kit (Takara Bio, Japan). The mRNA expression levels of COX-2, LOX-1, NOX2, and NOX4 in the aorta were determined by qRT-PCR using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The primer sequences used for qRT-PCR are shown in Table 1.

Table 1
Primer sequences used for quantitative real-time PCR.

Primer	Forward	Reverse
LOX-1	TGAAGCCTGCGAATGACGAG	GTCACTGACAACACCCAGGCAGAG
COX-2	TGCCAGGCTGAACTTCGAAAC	GCTCACGAGGCCACTGATACCTA
NOX-2	GCCCAAAGGTGTCCAAGC	TCCCCAACGATGCGGATAT
NOX-4	ACCCTGTTGGATGACTGGAA	ACCAACGGAAAGGACTGGATA
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

1.8. Western blot analysis

The aortas of the mice in the three groups were dissected and homogenized with ice-cold RIPA and 0.1 mmol/l of phenylmethylsulfonyl fluoride (PMSF) for 30 min. Total protein was isolated using the tissue protein extraction reagent (TPER; Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The concentration of total protein was determined using the Bio-Rad protein assay; 32 μg/lane of total protein was subjected to SDS-polyacrylamide gel (8%–10%) electrophoresis and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% BSA-TBST at 25 °C for 1 h, the membranes were washed and incubated with NOX2, NOX4, and NF-κB antibodies (Abcam, England) overnight at 4 °C and GAPDH in blocking buffer overnight at 4 °C. The membranes were then washed in fresh Tris-buffered saline containing Tween and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The reaction complexes in the membranes were detected using an enhanced chemiluminescence system (Jackson Lab, USA) and visualized using photographic Gel Image system films (ver. 4.00, Tanon, China). The signal intensities were automatically quantified by densitometric analysis using the Quantity One software (Bio-Rad, Hercules, CA, USA).

1.9. Statistical analysis

Data were collected and analyzed using the SPSS statistical software (version 13.0; SPSS Inc., Chicago, IL, USA). All data are expressed as mean ± standard deviation (SD). Comparative analyses between the three groups were performed using the t-test. A p-value of 0.05 was considered statistically significant.

2. Results

2.1. TSA reduced atherosclerotic plaque formation in the aortic sinus of the mice

Oil Red O staining revealed the presence of intense atherosclerotic plaques (lipid depositions) in cryosections from the aortic sinus (Fig. 2A). The percentage of the lumen in the proximal aorta occupied by the atherosclerotic plaque in the TSA-treated mice was significantly lower than that in the *P. gingivalis*-challenged mice (Fig. 2B; *P. gingivalis*: 9.89 ± 1.32% vs. *P. gingivalis* + TSA: 8.21 ± 1.47%, P < 0.05). On the contrary, the area of lipid deposition in the *P. gingivalis*-challenged group (9.87% ± 1.32%) was significantly higher (P < 0.05) than that in the PBS-treated group (5.44% ± 0.77%).

2.2. Decreased oxidative stress mediators in the sera of mice in the TSA-treated group

The expression levels of ROS, 8-OHdG and ox-LDL in the serum were evaluated to determine whether TSA regulated the oxidative stress during the early stages of AS. Significant decreases in the levels of ROS (Fig. 3A; *P. gingivalis*: 45.87 ± 3.90 IU/mL vs. *P. gingivalis* + TSA: 44.10 ± 2.78 IU/mL, P < 0.05), and ox-LDL (Fig. 3B; *P. gingivalis*: 7.55 ± 1.02 μmol/mL vs. *P. gingivalis* + TSA: 6.41 ± 0.90 μmol/mL; P < 0.05) were detected in the sera of the TSA-treated mice. The levels of 8-OHdG was slightly decreased in TSA-treated mice, although this did not reach statistical significance (Fig. 3C; *P. gingivalis*: 13.46 ± 3.65 ng/L vs. *P. gingivalis* + TSA: 10.29 ± 1.79 ng/L). Alternatively, the expression levels of ROS (Fig. 3A; *P. gingivalis*: 45.87 ± 3.90 IU/mL vs. PBS: 42.06 ± 2.84 IU/mL, P < 0.05), ox-LDL (Fig. 3B; *P. gingivalis*: 7.55 ± 1.02 μmol/mL vs. PBS: 5.25 ± 0.43 μmol/mL, P < 0.05), and 8-OHdG (Fig. 3C; *P. gingivalis*: 13.46 ± 3.65 ng/L vs. PBS: 9.56 ± 2.84 ng/L, P < 0.05) were significantly increased in the *P. gingivalis*-challenged group when compared to those in the PBS-controlled group.

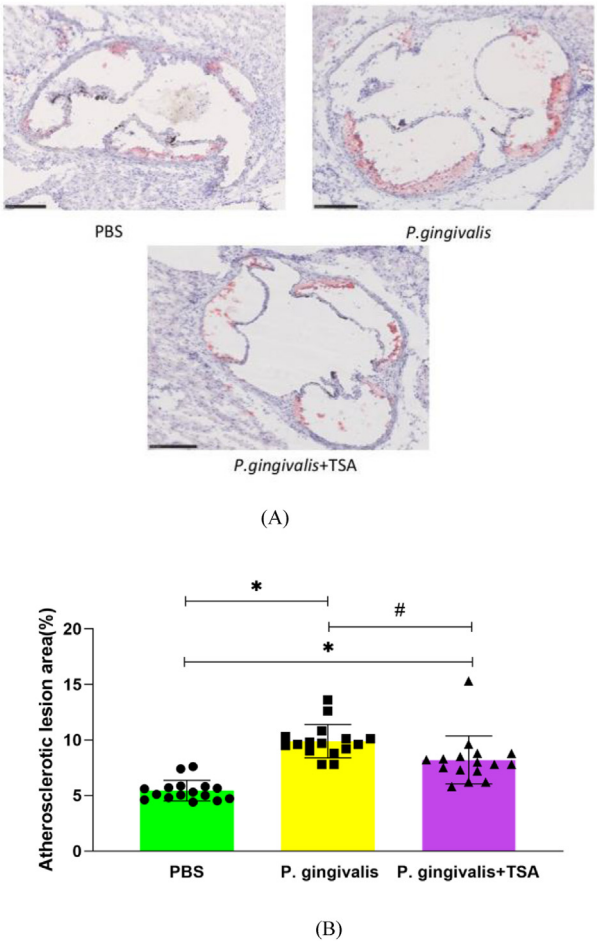

Fig. 2. Lipid deposition in the aortic sinus of ApoE−/− mice. (A). Lipid deposition stained with Oil Red O in aortic sinus. (B). The percentage of lesion areas was shown in the different group. The data represented the mean ± SD (n = 8). *P < 0.05 compared with the PBS-inoculated group; #P < 0.05 compared with the *Porphyromonas gingivalis*-challenged group.

2.3. TSA inhibited the mRNA expression of NOX2, NOX4, COX-2 and LOX-1 in the aorta

The mRNA levels of NOX2, NOX4, COX-2 and LOX-1 in the aorta were analyzed by qRT-PCR to determine the molecular mechanism involved in the NOX-mediated modulation of TSA. TSA decreased the mRNA levels of NOX2 (Fig. 3A; *P. gingivalis*: 1.27 ± 0.33 vs. *P. gingivalis* + TSA: 0.91 ± 0.31after normalization to GAPDH; P < 0.05) and NOX4 (Fig. 4B; *P. gingivalis*: 1.16 ± 0.41 vs. *P. gingivalis* + TSA: 0.74 ± 0.2; P < 0.05), which led to aggravation of AS. Furthermore, the superoxide levels of COX-2 (Fig. 4C; PBS:

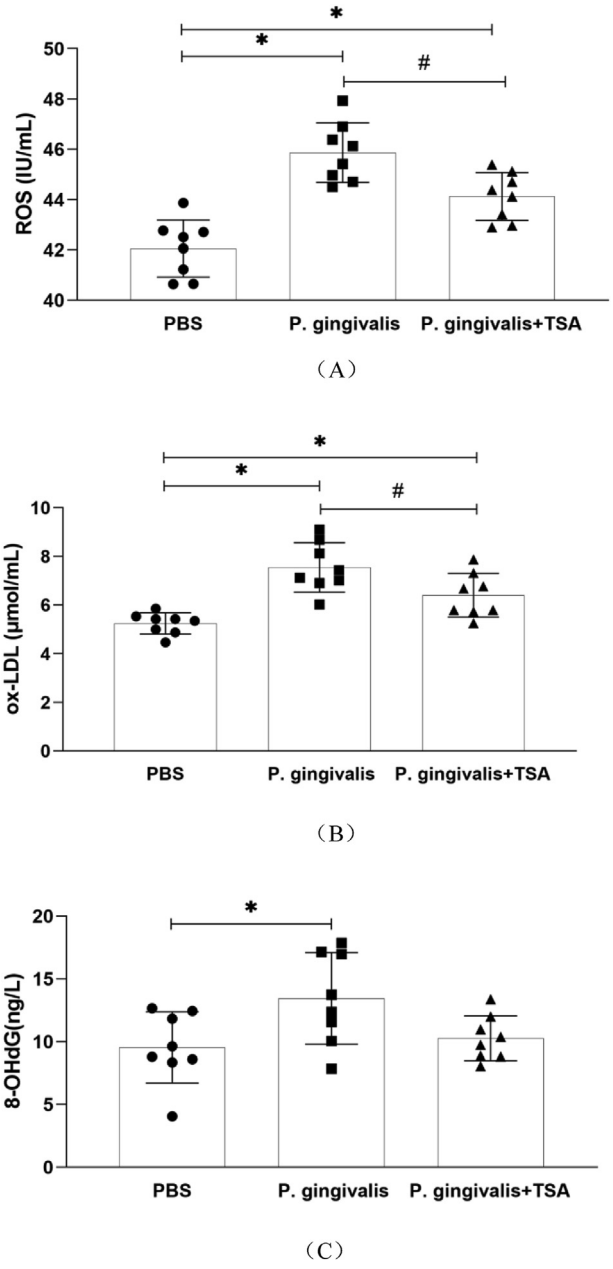

Fig. 3. ROS, 8-OHdG and ox-LDL in the serum detected by ELISA assay. ROS(A), ox-LDL(B) and 8-OHdG(C) in the serum were detected by ELISA assay. The data represented the mean ± SD (n = 8). *P < 0.05 compared with the PBS-inoculated group; #P < 0.05 compared with the *Porphyromonas gingivalis*-challenged group.

0.66 ± 0.34 vs. *P. gingivalis*: 1.31 ± 0.60; P < 0.05) and LOX-1 (Fig. 4D; PBS: 0.59 ± 0.23 vs. *P. gingivalis*: 1.48 ± 0.75; P < 0.05) were significantly increased in the *P. gingivalis*-challenged mice when compared to those in the PBS-controlled group. Thus, superoxide formation could be inhibited in the TSA-treated mice, suggesting that TSA alleviated oxidative stress to produce superoxide in the ApoE −/− mice.

2.4. NOX2, NOX4, and NF-κB expression contribute to TSA-alleviated AS in the aorta

Western blotting revealed significantly lower expression levels of NOX2 (Fig. 5A; *P. gingivalis* + TSA: 0.38 ± 0.08 vs. *P. gingivalis*: 0.50 ± 0.08; P < 0.05), and NF-κB (Fig. 5C; *P. gingivalis* + TSA 0.35 ± 0.04 vs. *P. gingivalis*: 0.45 ± 0.10; P < 0.05) in the aortas of the mice in the TSA-treated group compared to those in the *P. gingivalis*-infected groups. TSA-treated group decreased the proportion of NOX4 compared to those found in *P. gingivalis*-infected groups, although no statistical difference was observed between those groups (Fig. 5B; *P. gingivalis* + TSA 0.40 ± 0.08 vs. *P. gingivalis*: 0.50 ± 0.08). Additionally, significant reductions in the expression levels of NOX2 (PBS: 0.35 ± 0.05 vs. *P. gingivalis*: 0.50 ± 0.08; P < 0.05), NOX4 (PBS: 0.30 ± 0.07 vs. *P. gingivalis*: 0.50 ± 0.08; P < 0.05), and NF-κB (PBS: 0.30 ± 0.05 vs. *P. gingivalis*: 0.45 ± 0.10; P < 0.05) were observed in the PBS-incubated groups compared to the *P. gingivalis*-infected groups. The results were expressed as the ratio of the relative levels of NOX2, NOX4, NF-κB after normalization to GAPDH (Fig. 5D).

3. Discussion

AS is a chronic and multifactorial inflammatory cardiovascular disease with a high prevalence in developing countries and a mortality rate of 50% in developed countries. It is predicted as the leading cause of unstable angina, myocardial infarction, sudden cardiac death, and stroke; moreover, it is considered as one of the most serious diseases threatening human health across the world [1]. AS is initiated by endothelial dysfunction and accompanied by the accumulation of ox-LDL, which is altered by ROS. This results in the deposition of atherogenic plaques, which leads to the narrowing of the lumen and thickening of the vessel walls, eventually leading to thrombosis [14]. The pathogenesis of AS is very complex; it includes the lipid metabolism theory, oxidative stress theory, endothelial dysfunction theory, and chronic inflammation theory [2,15]. Nonetheless, the pathogenesis of this disease has not been fully illustrated, and effective measures for its prevention and treatment have not been taken so far.

P. gingivalis, a Gram-negative and anaerobic bacterium, is one of the most important pathogens associated with chronic periodontitis. It can cause initial inflammatory response in the periodontal tissue, activate the host immune response to produce inflammatory mediators, such as cytokines, chemokines, and adhesion molecules, and eventually lead to alveolar bone resorption. Studies have shown that *P. gingivalis* is an important risk factor that can be detected in the atherosclerotic plaque and accelerate the development of AS [16].

Chiu et al. [17] firstly detected the periodontal pathogens *P. gingivalis* and *Streptococcus haematococcus* in atherosclerotic plaques during artery bypass grafting. Haraszthy et al. demonstrated the presence of periodontal pathogens, including *Forsytans, Actinobacillus actinomycetemcomitans*, and *P. gingivalis,* in carotid artery plaques using the 16S rDNA technology [18], which opened up a new direction of research on the correlation between periodontitis and AS. In recent years, epidemiological studies have shown that periodontitis is an independent risk factor for AS [19]. The severity of AS increases with the increase in inflammation during periodontitis. However, the biological basis and mechanisms involved in this association between periodontitis and AS remain poorly understood. Clinical and animal studies have shown that *P. gingivalis* infection-mediated immune inflammatory response causes periodontitis and is also closely related to the occurrence and development of AS [20,21].

The virulence factors of *P. gingivalis,* such as lipopolysaccharides (LPS), proteases, and pili, could induce an immune inflammatory response, activate the movement of inflammatory cells into the blood circulation, and activate endothelial cells to release vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), P-promotes, E-promotes, monocytechemoattract antprotein-1 (MCP-1), interleukin (interleukin)-1β), IL-6, and IL-8

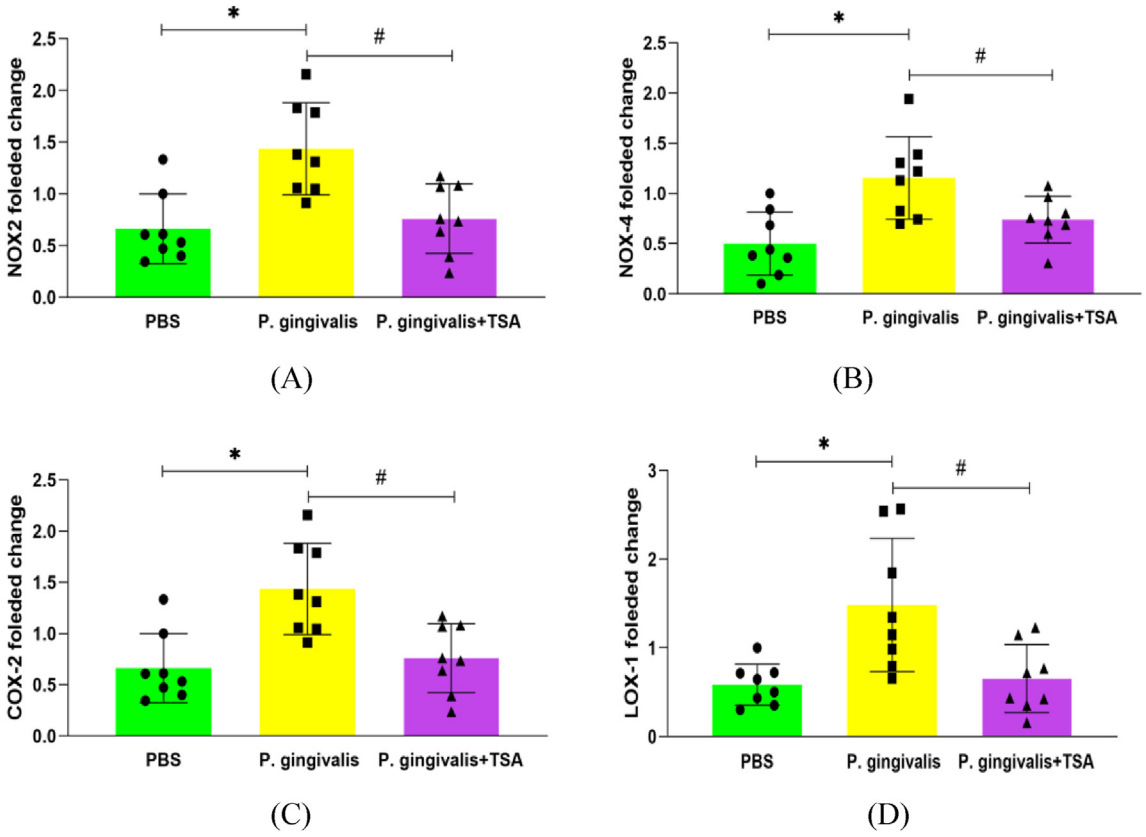

Fig. 4. mRNA expressions of NOX2, NOX4, COX-2, and LOX-1 obtained by quantitative real-time PCR. The relative mRNA expressions of NOX2(A), NOX4(B), COX-2(C), and LOX-1(D) were obtained after normalization to GAPDH by real-time PCR in aorta. Data are expressed as the fold increases in the mRNA level compared with the PBS-inoculated group or *Porphyromonas gingivalis*-challenged group. Values represent the mean ± SD (n = 8). *P < 0.05 compared with the PBS-inoculated group; #P < 0.05 compared with the *Porphyromonas gingivalis*-challenged group.

to promote the migration of vascular smooth muscle cells [3]. In our previous study, a large number of neutrophils and macrophages were detected in the aortic sinus of animal models of AS infected by *P. gingivalis* [4]. The bacteria could accelerate the development of AS, possibly by mediating oxidative stress and inflammatory responses and disturbing the lipid profile by increasing the vLDL and ox-LDL levels. Additionally, an increase in the expression levels of pro-inflammatory cytokines (C-reactive protein (CRP), IL-1β, IL-6, and tumor necrosis factor-α (TNF-α)) was observed in the serum along with the NF-κB signaling pathway in *P. gingivalis*-induced AS. NF-κB is one of the most important transcription factors involved in the regulation of several cytokines, chemokines, adhesion molecules, and growth factors [22]. It plays a pivotal role by regulating several inflammatory cytokines. Oxidative stress and inflammation are interrelated and work in a vicious feed-forward cycle during atherogenesis. ROS activates transcription factors which regulate inflammatory cytokines, chemokines, and soluble mediators, including arachidonic acid metabolites [23]. Cytokines and chemokines secreted by inflammatory cells recruit more inflammatory cells to the sites of inflammation, thereby increasing the production of ROS and exacerbating the adverse cycle [24].

Oxidative stress is one of the initiating factors in the development of AS, which is marked by elevated levels of ROS. ROS is an important signaling molecule that regulates the vascular structure and function; excessive ROS production by endothelial, vascular smooth muscle, and extracellular membrane cells is one of the risk factors for AS [25]. Accordingly, the ROS-producing enzymes include NADPH oxidase, xanthine oxidase, and cyclooxygenase. A variety of enzymes are involved in the production of ROS in the body, such as NADPH oxidase, lipoxygenase, the mitochondrial respiratory chain enzyme complex, and nitric oxide synthase. NADPH oxidase is the main enzyme that generates ROS in blood vessels. The inappropriate regulation of NOX contributes to inflammation and oxidative stress in AS [26].

The NOX family is a homolog of NADPH oxidase, which exists in different non-phagocytic cytoplasmic membranes. The expression of the seven different isoforms of NOX, including NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, DUOX2, is cell- and tissue-specific. Excessive activation of NOX is essential for the increase in oxidative stress and is closely related to the occurrence and development of many diseases. NOX2 is mainly expressed in phagocytic, endothelial, and arterial extracellular membrane cells, and its physiological functions mainly include immune defense, oxygen sensors and blood pressure regulation. NOX4 is primarily expressed in the kidney, endothelial cells, heart, skeletal muscle, and brain, and its physiological functions involve the regulation of erythropoietin synthesis and oxygen receptors. NOX2 and NOX4 are highly expressed in the cardiovascular system, particularly in endothelial cells and vascular smooth muscle cells (VSMCs) [27]. ROS-producing NOX2 has been detected in the endothelium and outer membrane, and NOX1 and NOX4 are essential for the function of VSMCs. The expression and activation of NOX4 results in enhanced cellular and mitochondrial oxidative stress, vascular inflammation, dysfunction, and AS. In addition, NOX4 expression has been associated with cellular proliferation, differentiation, and oxygen sensing [26]. Our previous study showed that chronic intravenous infection with *P. gingivalis* significantly accelerated the development of AS in the aortic sinus and increased the mRNA levels of

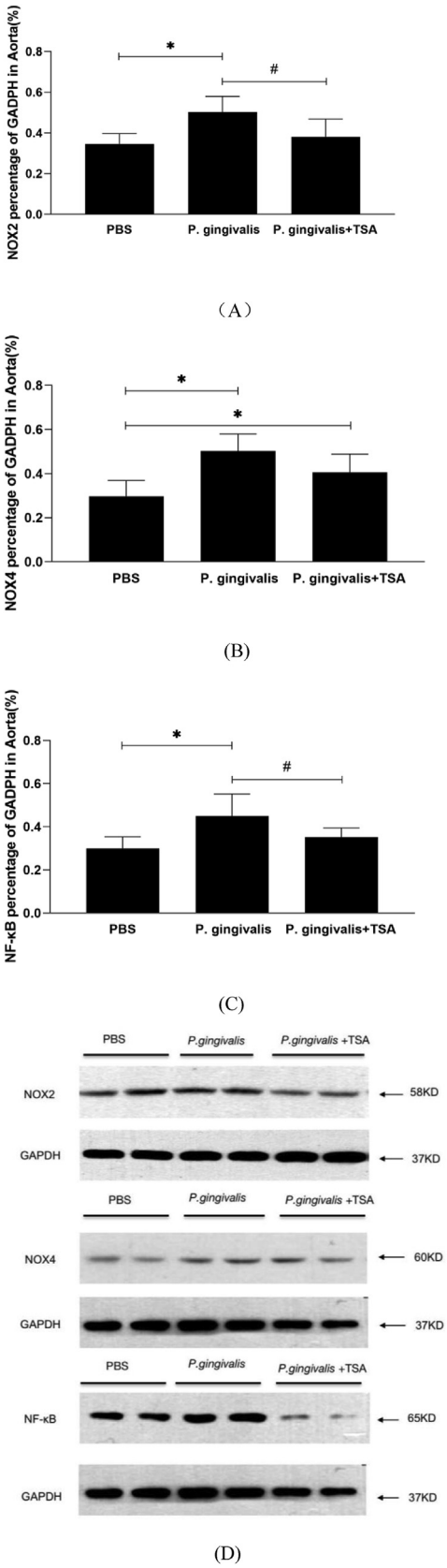

Fig. 5. NOX2, NOX4, and NF-κB analyzed by Western blotting. The expression of NOX2, NOX4, and NF-κB were analyzed by Western blotting in PBS-inoculated, *Porphyromonas gingivalis*-challenged, and TSA-treated mice. Data are expressed as the fold change increases in the *P. gingivalis*-challenged group compared with the PBS-inoculated group, and decreases in the TSA-treated group after normalization to GAPDH. Values represent the mean ± SD (n = 8). *P < 0.05 compared with the PBS-inoculated group; #P < 0.05 compared with the *Porphyromonas gingivalis*-challenged group.

NOX2 and NOX4 in the heart and aorta [5]. Therefore, inhibition of NOX could be promising for the prevention and treatment of AS [28].

TSA is a safe, effective, and natural active ingredient purified from the root of the *S. miltiorrhiza* Bunge used in traditional Chinese medicine. It exhibits anti-tumor, anti-oxidation, anti-inflammation, and antibacterial effects along with strong pharmacological activities in the cardiovascular system and nervous system [29]. TSA prevents atherosclerotic vascular disease by reducing the apoptosis of endothelial and smooth muscle cells, inhibiting platelet aggregation, and protecting the endothelium from damage. Studies have shown that TSA inhibits LPS-induced inflammation and NF-κB activity by blocking the TLR-4 pathway and reducing the expression of inflammatory cytokines, respectively [30]. In our previous study, we showed that TSA significantly reduced plaque formation and the levels of CRP, ox-LDL, IL-1β, IL-6, IL-12, matrix metalloproteinase (MMP)-2 and −9, and TNF-α by exerting its anti-inflammatory effects [13]. Furthermore, TSA decreased the levels of ox-LDL, COX-2, and LOX-1 via anti-oxidative stress. Thus, these factors may be crucial for the induction of AS by *P. gingivalis* in ApoE −/− mice.

TSA can affect multiple targets and pathways, including transcription factors, scavant receptors, cytokines and chemokines, ion and water channels, pro-apoptotic and anti-apoptotic proteins, growth factors, and microRNA [31,32]. Some studies have shown that TSA significantly inhibited oxidative stress by inhibiting the expression of NOX4 in cardiac fibrosis mice [33]. Others suggested that TSA protected the cardiac function from LPS challenge via the down-regulation of the NOX2-related ERK1/2 and p38 MAPK signaling pathways and ROS [34]. Over the past decades, some studies focused on the role of TSA in the prevention of LPS-induced cardiac dysfunction via the inhibition of inflammatory responses and oxidative stress [34–36]. However, the functional targets and molecular mechanisms involved in these biological effects remain elusive. Based on the findings of these various studies, we speculated that the anti-inflammatory effect and anti-oxidative stress of TSA may be related to the inhibition of the NOX family, which might be mediated by the expression of ROS.

Our present study demonstrated that the overexpression of NOX2 and NOX4 in the ApoE −/− mice model infected with *P. gingivalis* was associated with the increase in NF-κB and ROS in atherosclerotic lesions. These results suggested that ROS, which are derived from numerous sources such as mitochondria, xanthine oxidases, and peroxidases, could lead to the development of oxidative stress [37], which might be closely linked to the pathogenesis and progression of *P. gingivalis*-induced AS. TSA attenuates *P. gingivalis*-associated AS due to its inhibitory effects on NOX2 and NOX4 mediated by NF-κB and ROS. The NOX2, 4/ROS/NF-κB pathway is closely related to the regulation of inflammatory and oxidative stress responses during the course of AS. NOX2 and NOX4 in the vascular wall catalyze ROS production, which mediates oxidative stress and NF-κB activation, thereby mediating inflammation. This results in the stimulation of the expression of NOX2 and NOX4 leading to amplification of the oxidative stress response. Ultimately, the NOX/ROS/NF-κB pathway forms a mutually reinforcing positive feedback loop that significantly activates the inflammatory and oxidative stress responses [38,39]. In brief, our data suggested that *P. gingivalis*-induced AS up-regulated oxidative stress genes, including NOX2, NOX4, and NF-κB, while TSA down-regulated NOX2, NOX4, and NF-κB expression.

Some studies have indicated that the overexpression of NOX2 and NOX4 in the aorta reduced AS [40,41]. This protection is mediated by the up-regulation of T regulatory cells, which are known to prevent AS, and the down-regulation of T effector cells, which are involved in inflammatory responses [42]. The different effects of NOX4 may be related to the types of ROS produced by these enzymes. NOX4 produces hydrogen peroxide, which is localized in the nucleus, focal adhesions, and endoplasmic reticulum [43]. Under stress conditions, NOX4 localization in the mitochondria may promote mitochondrial oxidative stress, increase the levels of mitochondrial ROS, and activate both NF-κB and inflammatory signaling pathways.

The antibacterial effects of 95% alcohol *S. miltiorrhiza* against *P. gingivalis*, *A. actinomycetemcomitans*, *S. mutans,* and *Lactobacillus* have been reported previously [44]. These effects have been attributed to the inhibitory effect of TSA on RAW264.7 cells activated by LPS to synthesize NF-κB and its gene promoter, inhibition of inflammatory mediators and inflammation reaction [45]. These findings are consistent with those observed in the current study.

TSA attenuated the development of *P. gingivalis*-induced AS in the present study. The vascular protection afforded by TSA was associated with the reduction in the expression levels of NOX2 and NOX4, which was mediated by ROS and NF-κB. This study demonstrated that TSA might possess anti-inflammatory and antioxidative properties, which may decrease the deposition of lipids in the aorta, thereby having a therapeutic value in the treatment of cardiovascular and inflammatory diseases.

4. Conclusion

Given the complexity of the pathogenesis of AS, this study introduced the use of a knockout AS animal model. TSA was found to protect endothelial cells, prevent the formation of foam cells, reduce the expression of oxidative stress mediators and inflammatory cytokines, and intervene with multiple AS links via different pathways. Thus, it might be considered as a promising natural antioxidative and anti-inflammatory agent with pleiotropic effects. Additionally, this study provides information about the potential mechanism involved in *P. gingivalis*-induced AS and a possible pathway involved in preventing the further progression of this disease.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by Grants by the National Natural Science Foundation of China (No. 30320170990).

References

[1] Scott J. The pathogenesis of atherosclerosis and new opportunities for treatment and prevention. J Neural Transm Suppl 2002;(63):1–17.
[2] Perrotta I, Aquila S. The role of oxidative stress and autophagy in atherosclerosis. Oxid Med Cell Longev 2015:130315. 2015.
[3] Bale BF, Doneen AL, Vigerust DJ. High-risk periodontal pathogens contribute to the pathogenesis of atherosclerosis. Postgrad Med 2017 Apr;93(1098):215–20.
[4] XuanY ShiQ, LiuGJ LuanQX, CaiY. *Porphyromonas gingivalis* infection accelerates atherosclerosis mediated by oxidative stress and inflammatory responses in ApoE-/- mice. Clin Lab 2017 Oct 1;63(10):1627–37.
[5] Olsen I, Taubman MA, Singhrao SK. *Porphyromonas gingivalis* suppresses adaptive immunity in periodontitis, atherosclerosis, and Alzheimer's disease. J Oral Microbiol 2016 Nov 22;8:33029.
[6] Han X, Lin X, Yu X, Lin J, Kawai T, LaRosa KB, et al. Porphyromonas gingivalis infection-associated periodontal bone resorption is dependent on receptor activator of NF-κB ligand. Infect Immun 2013 May;81(5):1502–9.
[7] Senoner T, Dichtl W. Oxidative stress in cardiovascular diseases: still a therapeutic target? Nutrients 2019 Sep 4;11(9).
[8] Min Z, Yangchun L, Yuquan W, Changying Z. Quercetin inhibition of myocardial fibrosis through regulating MAPK signaling pathway via ROS. Pak J Pharm Sci 2019 May;32(3 Special):1355–9.
[9] Anilkumar N, Weber R, Zhang M, Brewer A, Shah AM. Nox4 and Nox2 NADPH oxidases mediate distinct cellular redox signaling responses to agonist stimulation. Arterioscler Thromb Vasc Biol 2008;28:1347–54.
[10] Octavia Y, Brunner-La Rocca HP, Moens AL. NADPH oxidase-dependent oxidative stress in the failing heart: from pathogenic roles to therapeutic approach. Free Radic Biol Med 2012;52:291–7.
[11] Huang L, Zhu J, Zheng M, Zou R, Zhou Y, Zhu M. Tanshinone IIA protects against subclinical lipopolysaccharide induced cardiacfibrosis in mice through inhibition of NADPH oxidase. Int Immunopharm 2018 Jul;60:59–63.
[12] Chen Z, Xu H. Anti-inflammatory and immunomodulatory mechanism of tanshinone IIA for atherosclerosis. Evid Based Complement Alternat Med 2014;2014:267976.
[13] Xuan Y, Gao Y, Huang H, Wang X, Cai Y, Luan QX. Tanshinone IIA attenuates atherosclerosis in apolipoprotein E knockout mice infected with Porphyromonas gingivalis. Inflammation 2017 Oct;40(5):1631–42.
[14] Gimbrone Jr MA, García-Cardeña G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circ Res 2016 Feb 19;118(4):620–36.
[15] Libby P. Inflammation in atherosclerosis. Nature 2002 Dec 19-26;420(6917):868–74.
[16] Henderson B, Kaiser F. Bacterial modulators of bone remodeling in the periodontal pocket. Periodontol 2000;76(1):97–108. 2018 Feb.
[17] Chiu B. Multiple infections in carotid atherosclerotic plaques. Am Heart J 1999;138(5 Pt 2):S534–6.
[18] Haraszthy VI, Zambon JJ, Trevisan M, et al. Identification of periodontal pathogens in atheromatous plaques. J Periodontol 2000;71(10):1554–60.
[19] Olsen I, Yilmaz Ö. Modulation of inflammasome activity by Porphyromonas gingivalis in periodontitis and associated systemic diseases. J Oral Microbiol 2016 Feb 4;8:30385.
[20] Wada K, Kamisaki Y. Roles of oral bacteria in cardiovascular diseases—from molecular mechanisms to clinical cases: involvement of Porphyromonas gingivalis in the development of human aortic aneurysm. J Pharmacol Sci 2010;113(2):115–9.
[21] Jain A, Batista Jr EL, Serhan C, et al. Role for periodontitis in the progression of lipid deposition in an animal model. Infect Immun 2003;71(10):6012–8.
[22] Prescott JA, Mitchell JP, Cook SJ. Inhibitory feedback control of NF-κB signalling in health and disease. Biochem J 2021-07-16;478(13):2619–64.
[23] Martinon F. Signaling by ROS drives inflammasome activation. Eur J Immunol 2010;40:616–9.
[24] Reuter S, Gupta S, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med 2010;49:1603–16.
[25] Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vasc Pharmacol 2018 Jan;100:1–19.
[26] Zhang Yixuan, Priya Murugesan, Huang Kai, Cai Hua. NADPH oxidases and oxidase crosstalk in cardiovascular diseases: novel therapeutic targets. Nat Rev Cardiol 2020 Mar;17(3):170–94.
[27] García-Redondo Ana B, Aguado Andrea, M Briones Ana, Salaices Mercedes. NADPH oxidases and vascular remodeling in cardiovascular diseases. Pharmacol Res 2016 Dec;114:110–20.
[28] Peng Rou, Luo Mengjuan, Tian Rong, Lu Naihao. Dietary nitrate attenuated endothelial dysfunction and atherosclerosis in apolipoprotein E knockout mice fed a high-fat diet: a critical role for NADPH oxidase. Arch Biochem Biophys 2020 08 15;689:108453.
[29] Ning Jian, He Chun-ling, Huang Jian-jun. Anti-inflammatory effect of tanshinone IIA in vitro and in vivo. Drugs & Clinic 2019;34(2):292–8.
[30] Meng Z, Si CY, Teng S, Li HY. Tanshinone IIA inhibits lipopolysaccharide-induced inflammatory responses through the TLR4/TAK1/NF-κB signaling pathway in vascular smooth muscle cells. Int J Mol Med 2019 Apr;43(4):1847–58.
[31] Li Z, Zou J, Cao D, Ma X. Pharmacological basis of tanshinone and new insights into tanshinone as a multitarget natural product for multifaceted diseases. Biomed Pharmacother 2020-10-01;130:110599.
[32] Wang X, Yang Y, Liu X, Gao X. Pharmacological properties of tanshinones, the natural products from Salvia miltiorrhiza. Adv Pharmacol 2020-01-01;87:43–70.
[33] Zhong Chenhui, Lin Zuan, Ke Liyuan, Shi Peiying, Li Shaoguang, Huang Liying, et al. Recent research progress (2015–2021) and perspectives on the pharmacological effects and mechanisms of tanshinone IIA. Front Pharmacol 2021;12:778847.
[34] Chen R, Chen W, Huang X, Rui Q. Tanshinone IIA attenuates heart failure via inhibiting oxidative stress in myocardial infarction rats. Mol Med Rep 2021 06;(6):23.
[35] Liu QY, Zhuang Y, Song XR, Qiu ZX. Tanshinone IIA prevents LPS-induced inflammatory responses in mice via inactivation of succinate dehydrogenase in macrophages. Acta Pharmacol Sin 2021-06-01;42(6):987–97.

[36] Zhou D, Yang WK, Zhang YF, Xie P. Sodium tanshinone IIA sulfonate prevents radiation-induced damage in primary rat cardiac fibroblasts. Chin J Nat Medcines 2020-06-01;18(6):436–45.
[37] Incalza MA, D'Oria R, Natalicchio A, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vasc Pharmacol 2018;100:1–19. 01.
[38] Lu W, Kang J, Hu K, et al. The role of the Nox4-derived ROS-mediated RhoA/Rho kinase pathway in rat hypertension induced by chronic intermittent hypoxia. Sleep Breath 2017;21(3):667–77.
[39] Lozhkin A, Vendrov AE, Pan H, et al. NADPH oxidase 4 regulates vascular inflammation in aging and atherosclerosis. J Mol Cell Cardiol 2017;102:10–21.
[40] Drummond GR, Sobey CG. Endothelial NADPH oxidases: which NOX to target in vascular disease? Trends endocrin Met 2014-09-01;25(9):452–63.
[41] Gray SP, Di Marco E, Kennedy K, Jandeleit-Dahm KA. Reactive oxygen species can provide atheroprotection via NOX4-dependent inhibition of inflammation and vascular remodeling. Arterioscl Throm Vas 2016-02-01;36(2):295–307.
[42] Tse K, Tse H, Sidney J, Sette A, Ley K. T cells in atherosclerosis. Int Immunol 2013;25:615–22.
[43] Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2004;24:677–83.
[44] Deng J, Zhang DZ, Yang WJ. An in vitro experiment on the antimicrobial effects of ethanol extract from salvia miltiorrhiza bunge on several oral pathogenic microbes. Shang Hai Kou Qiang Yi Xue 2006 Apr;15(2):210–2.
[45] Jang SI, Kim HJ, Kim YJ, et al. Tanshinone IIA inhibits LPS-induced NF-kappaB activation in RAW 264.7 cells: possible involvement of the NIK-IKK, ERK1/2, p38 and JNK pathways. Eur J Pharmacol 2006 Aug 7;542(1–3):1–7.