

Neohesperidin Protects Hypertension-induced Endothelial Injury by Intervening Oxidative Stress

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Research

Keywords: Neohesperidin, Angiotensin II, oxidative stress, hypertension, HUVECs

Posted Date: June 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-553058/v1>

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Abstract

Background: Currently, vascular endothelial damage caused by hypertension is one of the major health challenges facing countries around the world. Neohesperidin has been shown to play an important role in tumorigenesis and tumorigenesis, cardiac hypertrophy and remodeling, and oxidative stress. However, whether Nehesperidin plays an important role in endothelial injury induced by hypertension has not been clarified.

Results: In this study, Angiotensin II was used to induce hypertension in mice. Blood pressure and vasoconstrictor function were measured, vascular thickness and fibrosis were detected by H&E and Masson tricolor staining, vascular inflammation was detected by immunofluorescence, oxidative stress was detected by DHE staining, and markers such as fibrosis, hypertrophy and oxidative stress were detected by qPCR. At the same time, we observed the effect of Nehesperidin on Ang II-induced HUVECs. The results showed that neohesperidin can significantly inhibit Ang II-induced hypertension, vascular thickness, fibrosis, oxidative stress and inflammation in vivo and in vitro.

Conclusions: The results suggested that Nehesperidin could act as an antioxidant to significantly inhibit Ang II-induced hypertension and endothelial injury in HUVECs in mice by inhibiting oxidative stress response.

Background

With the acceleration of the aging of human society, chronic diseases have become a major global public health problem. Hypertension, one of the chronic diseases with a high incidence, is the main inducement for the structural and functional lesions of the heart, blood vessels, brain and kidney, as well as the most important risk factor for cardiovascular diseases[1]. Hypertension is a multifactorial disease, which is the result of the dynamic interaction among genetic, physiological and environmental factors. The pathophysiology of hypertension involves many mechanisms, such as the up-regulation of the renin-angiotensin aldosterone system, the activation of the sympathetic nervous system, the interference of G-protein-coupled receptor signals, and vascular inflammation[2, 3].

In 1989, Baumbach and Heistad first proposed the concept of "vascular remodeling" in the study of hypertension[4]. Vascular remodeling in hypertension is mainly manifested as vascular structure and function changes such as thickening of vessel wall, increasing ratio of wall thickness to lumen diameter, and decreasing number of small arteries[5]. Currently, it is believed that the damage to the intima (endothelial dysfunction), the thickening of the media and the rearrangement of the matrix components of the outer membrane caused by hypertension are important factors in the occurrence of vascular remodeling, and also one of the main mechanisms to maintain and promote the further development of vascular remodeling [6–8].

Vascular endothelial cells are monolayer epithelial cells distributed on the inner surface of blood vessels. When blood pressure continues to rise, the shear stress on the vessel wall can directly affect the vascular

endothelial cells and cause mechanical damage, leading to endothelial cell dysfunction, even necrosis and exfoliation [9]. Hypertension can damage the endothelium, and likewise, damage to the endothelium can exacerbate an increase in blood pressure. Vascular endothelial cells (VECs) constitute the largest secretory organ in the human body and play a role in the regulation of vascular physiological functions through autocrine, paracrine and endocrine pathways. Vascular endothelium regulates blood pressure mainly by regulating the secretion of vasoactive factors. Endothelial cells continuously synthesize and secrete a variety of active factors, among which Prostacyclin₂ (PGI₂) and Nitric oxide (NO) play a major role in vasodilating, while Endothelin-1 plays a major role in vasoconstricting. Thromboxane A₂ (TXA₂), Angiotensin II (Ang II), superoxide anion and ET-1 (TXA₂) were the main constrictor factors of blood vessels. Ang II is the main active substance of renin-angiotensin system. It not only plays a key role in the acute and chronic regulation of systemic arterial blood pressure, but also is an important regulator of cardiovascular function [10, 11]. In hypertension the expression of ICAM-1 and E-selectin increased, and these adhesion molecules made granulocytes and monocytes in the bloodstream adhere to vascular endothelial cells and enter the subcutaneous space. At the same time, activated vascular endothelial cells synthesize monocyte chemoattractant protein-1 (MCP-1), which accelerates the migration process of monocyte, and makes the monocyte adhere to the endothelium easily pass through the endothelial space and migrate to the inner subcutaneous layer. Once inflammatory cells enter the vessel wall, they release highly reactive oxygen free radical molecules (such as superoxide anion), causing oxidative stress of vascular endothelial cells, and causing vascular endothelial cell dysfunction into a vicious cycle.

Under the stimulation of pathological factors such as hyperlipidemia, hyperglycemia, Reactive oxygen species (ROS) and high blood flow shear stress, vascular endothelial cells will have dysfunction [12, 13]. The cytotoxic process caused by ROS is called oxidative stress. In the cardiovascular system, ROS plays an important role in the control of endothelial function and vascular tone, and plays a pathophysiological role in inflammation, hypertrophy, proliferation, apoptosis, migration, fibrosis, and angiogenesis. In the vascular wall, ROS is involved in the regulation of endothelial-dependent function, proliferation and apoptosis of vascular smooth muscle cells and endothelial cells, and vascular wall remodeling [14, 15]. The most important ROS in blood vessels is the superoxide anion mentioned above, which can inactivate the vasodilator nitric oxide (NO), then damage the vasodilation function, cause endothelial dysfunction, promote the change of vascular tension, increase resistance, and lead to vascular remodeling and hypertension.

Neohesperidin is a flavonoid compound abundant in Cucurbitaceae, which has antioxidant and anti-inflammatory effects [16, 17]. We have been reported that Neohesperidin could inhibit cardiac remodeling induced by Ang II in vivo and in vitro [18]. Since vascular endothelial injury is also a kind of oxidative stress injury, whether Neohesperidin can also alleviate the endothelial injury caused by Ang II by inhibiting oxidative stress? This paper is to test this hypothesis.

Results

Neohesperidin inhibits hypertension induced by Ang II in mice

We randomly divided the mice into four groups (Saline, Neohesperidin, Ang II and Ang II+Neohesperidin). Neohesperidin was injected into the caudal vein two days earlier. Mice were gavaged with Ang II (490ng/kg/min) and blood pressure was measured every two days. The flow chart is shown in Fig. 1A. We measured blood pressure and found that blood pressure of mice treated with Ang II was significantly higher than that of the control group. Moreover, after Neohesperidin treatment, the blood pressure of mice was relieved, which was statistically significant. This indicated that the model of hypertension in mice was successfully established (Fig.1B).

Inhibition of Neohesperidin can prevent endothelial dysfunction induced by Ang II in mice

The experimental methods for detecting vascular endothelial function generally include Vascular relaxation examinations. In this paper, we investigate whether Neohesperidin can improve vascular dysfunction. The results showed that Ang II significantly reduced the effect of ACh on endothelium-dependent vasodilation compared with saline control, while Neohesperidin significantly preserved vasodilation (Fig.2A). But no significant difference was observed in endothelium-dependent vasodilation of SNPs among the four groups (Fig.2B). Therefore, inhibition of Neohesperidin can prevent endothelial dysfunction induced by Ang II.

Neohesperidin alleviates Ang II-induced vascular hypertrophy and fibrosis

Vascular hypertrophy and sclerosis are the main pathological basis of complications of heart, brain and kidney caused by hypertension[19]. In this paper, the thickness of blood vessels in each group was measured by H&E staining. The results showed that the blood vessel thickness of Ang II group was significantly thickened, while the blood vessel thickness of Ang II group was significantly reduced after Neohesperidin treatment (Fig.2A). Ang II induces vascular dysfunction, promotes tissue fibrosis and remodeling, and is an important pathogenesis of many diseases such as hypertension, coronary heart disease, cerebrovascular disease and renal failure. Similarly, in this paper, we found that after Ang II induction, the degree of vascular fibrosis and the mRNA levels of related factors (Collagen I, Collagen III and α -SMA) in mice were significantly increased, while after Neohesperidin treatment, all indicators were decreased (Fig.3B).

Neohesperidin alleviates Ang II-induced vascular inflammation and oxidative stress

Recent studies have found that inflammation and hypertension influence each other and cause and effect each other. Inflammation promotes hypertension by changing the biological activity of nitric oxide and reducing endothelium-dependent vasodilator factors. In this paper, we used immunofluorescence staining for CD68, and the results showed that the mRNA expression levels of CD68 and inflammation-related factors in blood vessels of Ang II group were significantly increased, while the mRNA expression levels of CD68 and related factors were significantly decreased after Neohesperidin treatment. Oxidative stress and inflammation are closely related. Oxidative stress can cause inflammation, which in turn affects oxidative stress. Both can lead to cellular damage, including endothelial dysfunction. Endothelial dysfunction contributes to the formation of an inflammatory environment. Similarly, in this paper, the mRNA expression levels of DHE and related factors in blood vessels of the Ang II group were significantly increased, while the mRNA expression levels of DHE and related factors were significantly decreased after Neohesperidin treatment.

Neohesperidin inhibits Ang II-induced HUVECs ROS and DNA Damage

We have shown in vivo that Neohesperidin can inhibit Ang II-induced hypertension in mice, and speculate that it may act by alleviating endothelial damage. So we're going to test that with in vitro experiment. Vascular endothelial injury is an important manifestation of vascular remodeling, which is mainly involved in oxidative stress. γ -H2AX and p-ATM are markers of cellular oxidative stress and DNA damage. First, we detected the expression number of γ -H2AX in each group's cell nucleus. The results showed that AngII could significantly promote the expression of γ -H2AX, and also promote the expression of γ -H2AX in each cell nucleus, while the addition of Neohesperidin inhibited this tendency (Figure 5A, B). The corresponding results are also obtained in p-ATM (Figure 5C, D). Oxidative stress is closely related to vascular endothelial injury. When MDA increases, the level of oxidation increases, while SOD and GSH-PX increase, the level of antioxidant increases. In this paper, we examined the expression levels of these three factors. The results showed that the expression of MDA increased and the expression of SOD and GSH-PX decreased after Ang II stimulation. This is consistent with the literature. Compared with Ang II group, the expression of MDA decreased while the expression of SOD and GSH-PX increased after adding Neohesperidin (Figure 5E-G). Next, the degree of ROS mRNA levels of related factors (NOX1, NOX2, NOX4 and p22^{phox}) in mice were significantly increased, while after Neohesperidin treatment, all indicators were decreased (Figure 5H)

Discussion

In recent years, the incidence of cardiovascular disease has been increasing, and it has surpassed cancer as the leading cause of death in Western countries. As a prominent disease in an aging society, hypertension is a common chronic disease that can cause a variety of cardiovascular and cerebrovascular dangerous events, such as myocardial infarction, heart failure, and cerebrovascular accidents[20]. It is reported that Ang II is involved in inflammatory response, cell growth, ECM deposition

and blood coagulation and other processes, and plays an important role in the process of regulating the structure and function of the blood vessel wall[21].

In this study, we used Ang II to create a mouse model of hypertension, and found that the mice's blood vessels were thickened, blood pressure increased, oxidative stress and inflammation indicators increased. Among these complex pathological mechanisms, there is a common cause that is the increased availability of reactive oxygen species (ROS), that is, the occurrence of oxidative stress.

In recent years, more and more pharmacological studies have found that Neohesperidin can inhibit myocardial hypertrophy and remodeling[18], inhibit allergic reactions[17], reduce pulmonary fibrosis[17], anti-aging[22], prolong the lifespan of *Saccharomyces cerevisiae*, inhibit tumor occurrence and development, and reduce nerve cells Apoptosis, improve osteoporosis and so on. Therefore, we hypothesized that Neohesperidin may be able to dare to damage the vascular endothelium by Ang II by inhibiting oxidative stress. The results show that Neohesperidin can indeed inhibit the increase in blood pressure, thickening of blood vessel walls and increased inflammation in mice.

Vascular endothelial cells are an important barrier in the vascular cavity. They not only have the function of maintaining hemodynamic stability and material exchange, but also secrete inflammatory factors and vasodilation and contraction factors, which play an important role in the regulation of blood pressure. The abnormality is one of the important factors that promote the occurrence and development of hypertension. Oxidative stress is an important cause of vascular endothelial damage[23]. In this article, in vitro experiments have found that Ang II can cause DNA damage and oxidative stress damage to HUVECs, and Neohesperidin can inhibit the oxidative stress damage caused by Ang II.

Conclusions

The above experiments confirmed that Neohesperidin has a protective effect on endothelial cells, can inhibit the endothelial damage induced by Ang II, and play a protective effect by regulating the changes of oxidative stress. Of course, the molecular mechanism of Neohesperidin's protection of endothelial cells is very complicated, and the pathways of other involved signals are still unclear. It is important to further explore Neohesperidin's protection of endothelial cells and regulating cell apoptosis to protect vascular function and prevent cardiovascular events.

Methods

Animals and treatment

Male C57BL/6 mice 24g aged from 8 to 10 weeks were selected. The mice were divided into SHAM, Neohesperidin, Ang II, Neohesperidin+Ang II. Mice were gavaged with Ang II (490ng/kg/min, Aladdin, Ca) by Alzetmodel 1002 micropump for 2 weeks[18]. A caudal vein injection of 50mg/kg/day was performed for 16 days. All the procedures involved in the mouse study were approved by the Institutional Animal Care and Use Committee of Dalian Medical University.

Blood pressure measurement

As described earlier, blood pressure was measured in awake mice using the tail-sleeve method. The ambient temperature is maintained at a warm room temperature (25-30 °C)[24].

Histopathological

The blood vessels were fixed in 4% paraformaldehyde for 1 day, then embedded and sectioned. Sections were stained with hematoxylin-eosin (H&E), Mason trichrome staining, immunofluorescence (CD68 antibody, 1:100) and dihydropyrimidine (DHE). The positive regions were analyzed using ImageJ[18].

Real-time PCR

The cDNA was used for PCR amplification and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The Sequences is shown in Table 1.

Table 1

Primers used for quantitative real-time PCR analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
m-IL-1 β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
m-IL-17	TCAGCGTGTCCAAACACTGAG	CGCCAAGGGAGTTAAAGACTT
m-TNF- α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
m-Collagen I	GCTCCTCTTAGGGGCCACT	ATTGGGGACCCTTAGGCCAT
m-Collagen III	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
m-NOX1	CCTGATTCCTGTGTGTCGAAA	TTGGCTTCTTCTGTAGCGTTC
m-NOX2	AGTGCGTGTTGCTCGACAA	GCGGTGTGCAGTGCTATCAT
m-NOX4	TGCCTGCTCATTGGCTGT	CCGGCACATAGGTAAAAGGATG
m- α -SMA	CCCAGACATCAGGGAGTAATGG	TCTATCGGATACTTCAGCGTCA
m-GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
h-NOX1	TTGTTTGGTTAGGGCTGAATGT	GCCAATGTTGACCCAAGGATTTT
h-NOX2	ACCGGGTTTATGATATTCCACCT	GATTTTCGACAGACTGGCAAGA
h-NOX4	CAGATGTTGGGGCTAGGATTG	GAGTGTTCCGGCACATGGGTA
p22 ^{phox}	CCCAGTGGTACTTTGGTGCC	GCGGTCATGTACTTCTGTCCC
h-GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Vascular relaxation examinations

The thoracic aorta was separated, and then gently installed on the force sensor (DK) in the organ cavity. After stimulation with norepinephrine, endothelium-dependent or endothelium-independent relaxation in response to increased concentrations of acetylcholine (ACh) or sodium nitroprusside (SNP) was recorded.

Human Umbilical Vein Endothelial Cells (HUVECs) culture

The neonatal umbilical cord was digested with 15ml collagenase (1mg/ml) at room temperature for 15-20 min, and the collected solution was centrifuged (2000 r/min) for 3 min. The supernatant was poured and added into 10ml M199 medium (with 10U/ml bFGF) for culture at 37°C. After incubation for 24 hours, pour out the medium and wash it with sterile PBS solution 2-3 times to wash off the red cellules and dead cells, and add 10 mL fresh M199 medium.

Malondialdehyde (MDA), Superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px)

All three kits were purchased from Solibao. After grouping treatment, the cells were lysed and determined on the enzyme plate analyzer according to the instructions.

Statistical analysis

All data were analyzed using GraphPad 8.0 software. Data were compared using one-way ANOVA, independent t-test, or chi-square test. * <0.05, ** <0.01, *** <0.001 is statistically significant compared with Saline, # <0.05, ## <0.01, ### <0.001 is statistically significant compared with Ang II.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics Statement

All experimental procedures involve in the mouse studies were approved by the Institutional Animal Care and Use Committee in Dalian Medical University.

Funding

This work was supported by the National Natural Science Foundation of China (81770617)

Acknowledgements

Not applicable.

Availability of data and materials

The data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Author Contributions

JZ, YH, FL, QY, YL and YC Investigation and acquisition of data, analysis and interpretation of data, visualization, study concept or design, and original draft of the manuscript. QL and YD revising the manuscript, funding acquisition, and study supervision. All authors contributed to the article and approved the submitted version.

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Figures

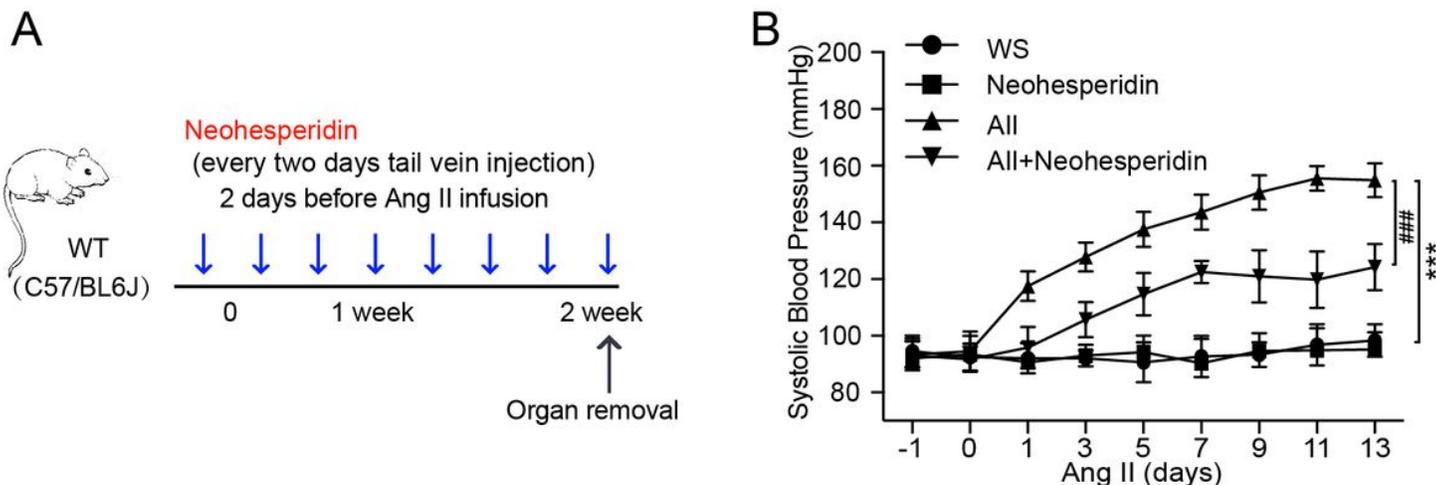


Figure 1

Figure 1

Neohesperidin inhibits hypertension induced by Ang II in mice. (A) Schematic diagram of the whole process of the experiment; (B) An average systolic blood pressure of each group. *** P<0.001, sham vs Ang II; ### P<0.001, Ang II vs Ang II+ Neohesperidin

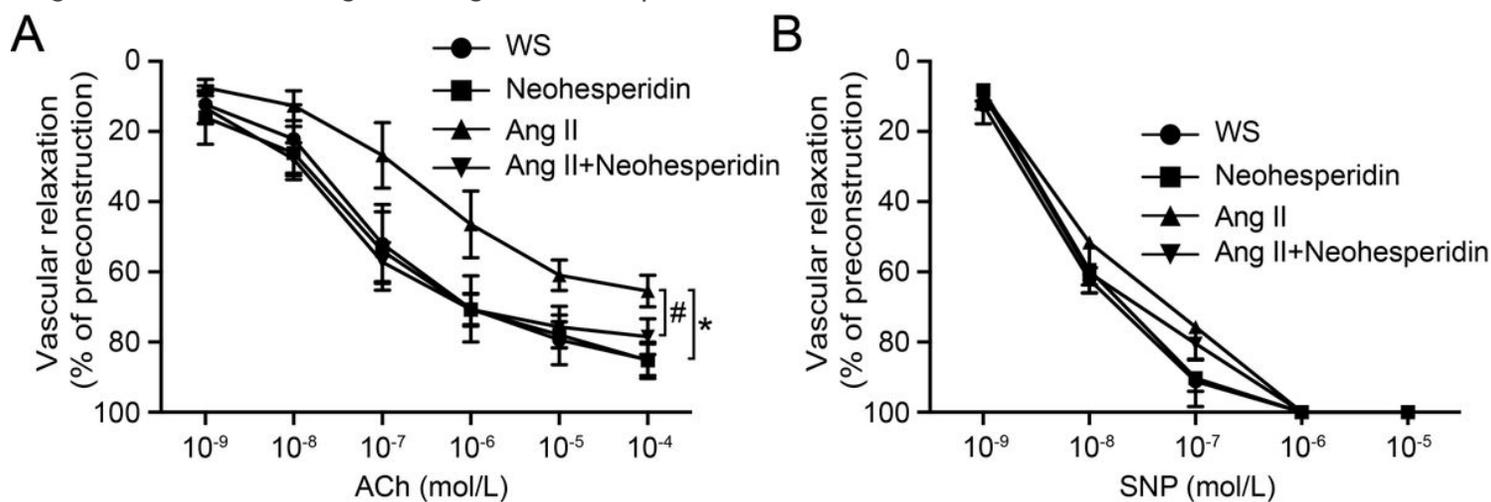


Figure 2

Figure 2

Inhibition of Neohesperidin can prevent endothelial dysfunction induced by Ang II in mice. Aortic rings were isolated from each group. (A) We examined endothelium-dependent relaxation in response to different doses of acetylcholine; (B) We examined endothelial independent relaxation in response to

different doses of sodium nitroprusside. * $P < 0.05$, sham vs Ang II; # $P < 0.05$, Ang II vs Ang II+ Neohesperidin

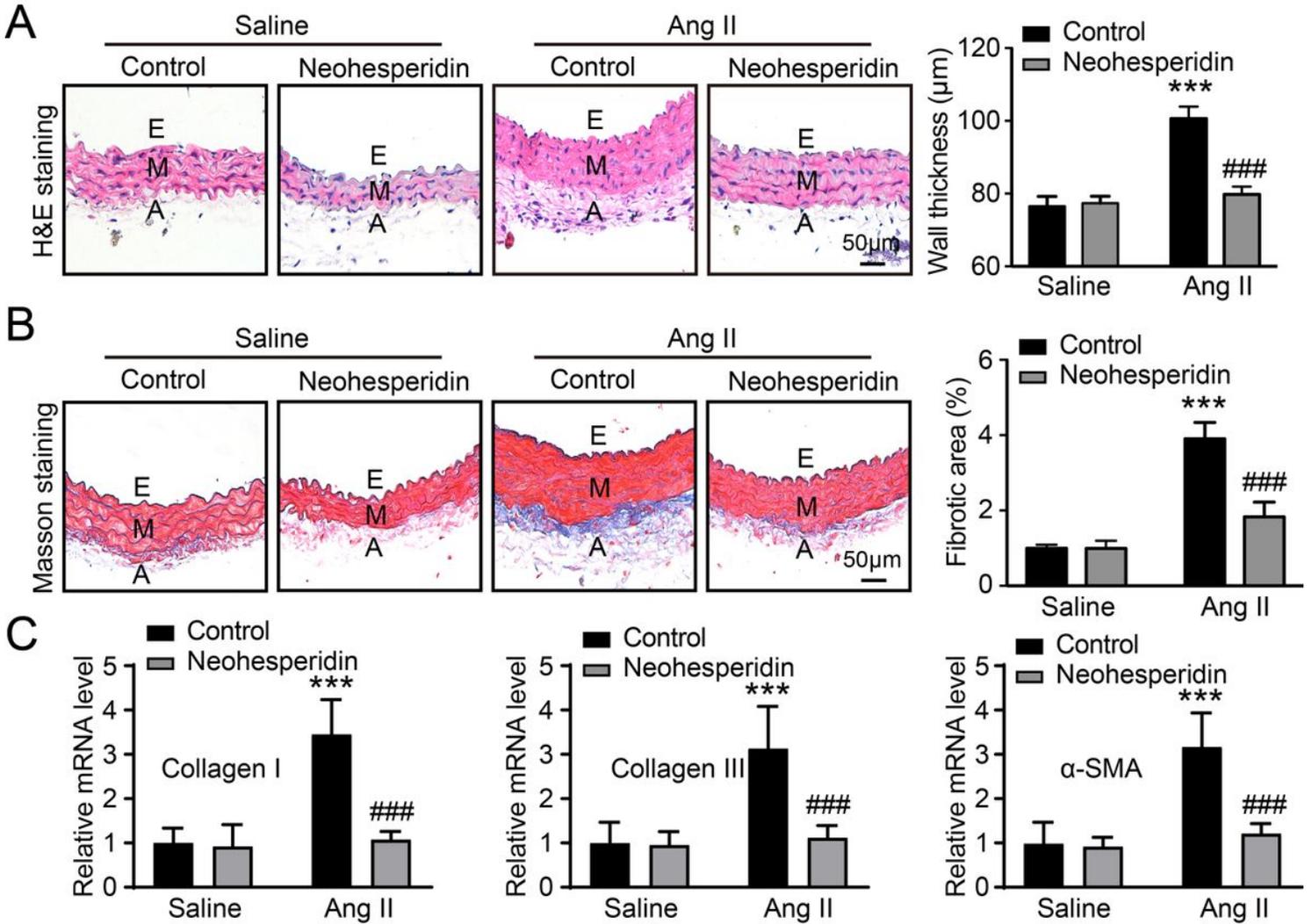


Figure 3

Figure 3

Neohesperidin alleviates Ang II-induced vascular hypertrophy and fibrosis (A) Representative images of H&E staining from each group (left panel, Bar=50 µm). Histogram of measured aortic wall thickness (right panel); (B) Representative images of Masson's trichrome staining from each group (left panel, Bar=50 µm). Q Histogram of measured fibrotic area (right panel); (C) qPCR analysis of collagen I, collagen III and α-SMA mRNA levels from each group. *** $P < 0.001$, sham vs Ang II; ### $P < 0.001$, Ang II vs Ang II+ Neohesperidin

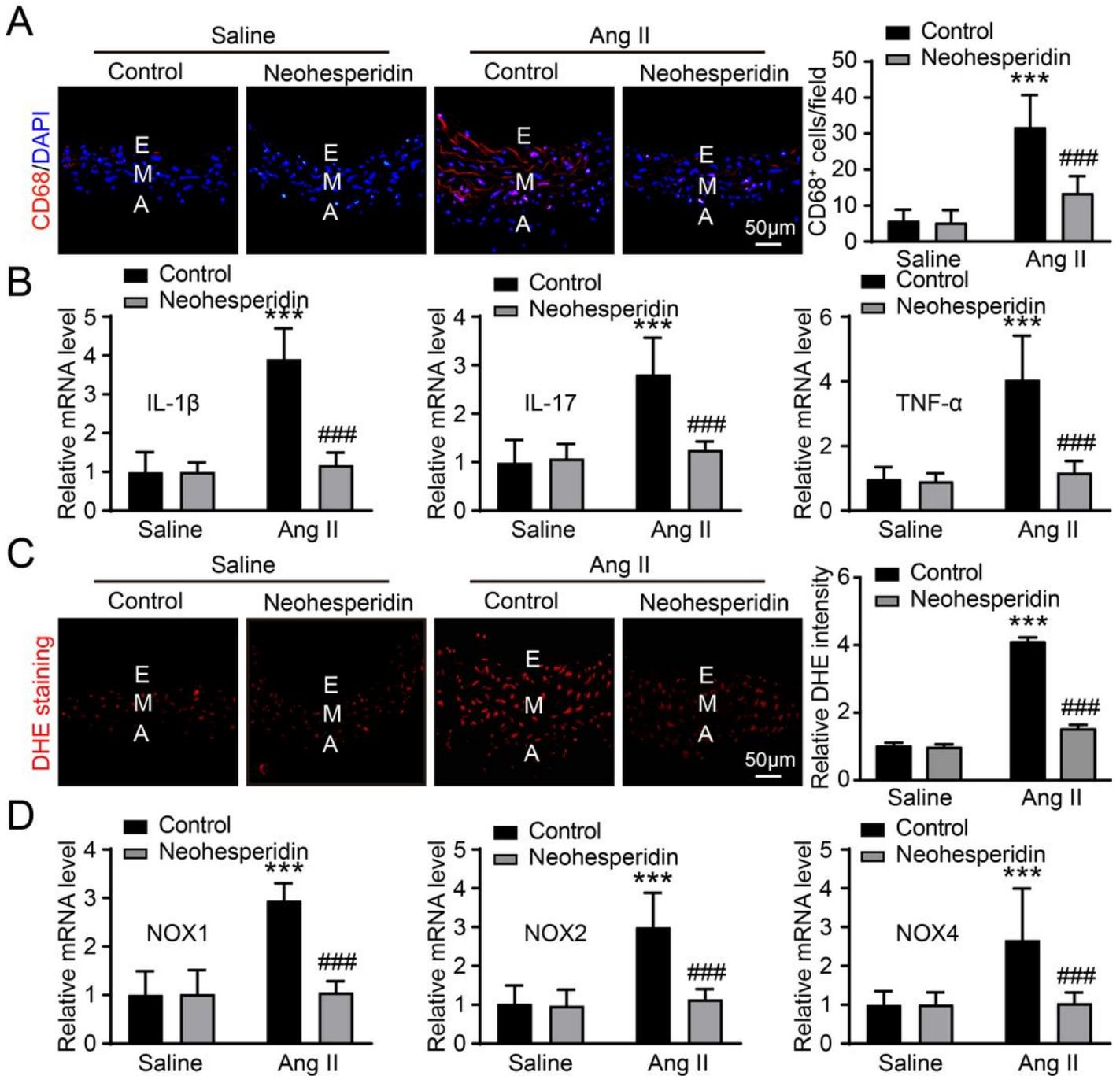


Figure 4

Figure 4

Neohesperidin alleviates Ang II-induced vascular inflammation and oxidative stress. (A) Representative images of CD68 staining from each group (left panel, Bar=50 μ m). Histogram of CD68⁺ cells (right panel); (B) qPCR analysis of IL-1 β , IL-17 and TNF- α mRNA levels from each group. (C) Representative images of DHE staining from each group (left panel, Bar=50 μ m). Histogram of relative DHE intensity

(right panel); (D) qPCR analysis of NOX1, NOX2 and NOX4 mRNA levels from each group. *** P<0.001, sham vs Ang II; ### P<0.001, Ang II vs Ang II+ Neohesperidin

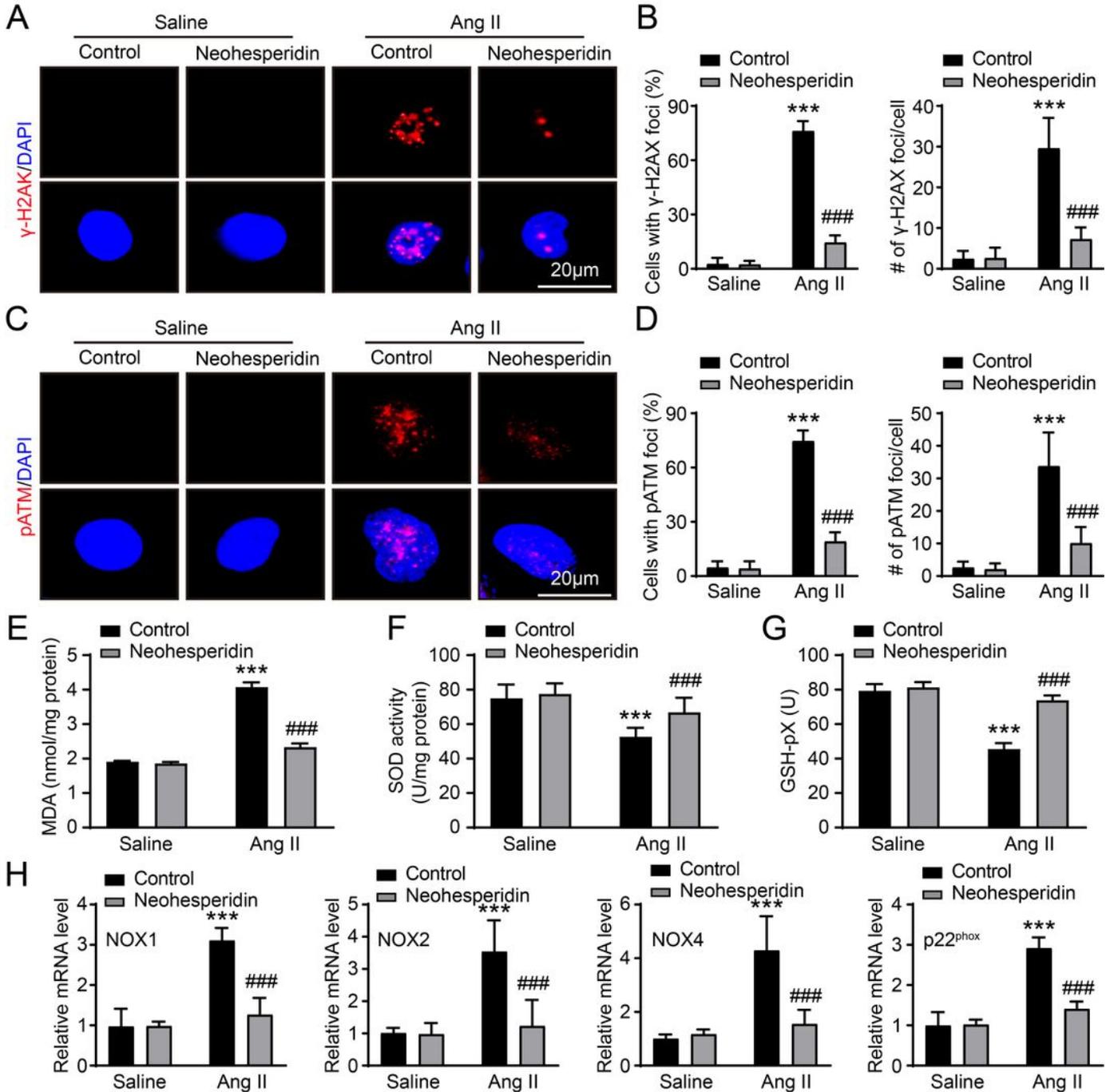


Figure 5

Figure 5

Neohesperidin inhibits Ang II-induced HUVECs ROS and DNA Damage (A) Representative images of γ -H2AX staining from each group (Bar=20 μ m); (B) Histogram of the percentage of HUVECs with positive γ -H2AX-positive nuclear foci (left) and the average number of γ -H2AX foci in γ -H2AX-positive HUVECs

(right); (C) Representative images of p-ATM staining from each group (Bar=20 μ m); (D) Histogram of the percentage of HUVECs with positive p-ATM -positive nuclear foci (left) and the average number of p-ATM foci in p-ATM -positive HUVECs (right); (E) Histogram of Malondialdehyde (MDA); (F) Histogram of Superoxide dismutase (SOD); (G) Histogram of Glutathione peroxidase (GSH-Px); (H) qPCR analysis of NOX1, NOX2, NOX4 and p22phox mRNA levels from each group. *** P<0.001, sham vs Ang II; ### P<0.001, Ang II vs Ang II+ Neohesperidin.