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Original Research

Canthaxanthin Attenuates the Vascular Aging or Endothelial Cell Senescence by Inhibiting Inflammation and Oxidative Stress in Mice

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Abstract

Background: Vascular endothelial dysfunction is an early phenotype of aging-related vascular dysfunction. Delaying vascular aging and preventing cardiovascular disease are major public health problems that urgently need to be solved. Scientists have studied various drugs to prevent the occurrence and progress of cardiovascular disease, but progress has been slow. Here, the antisenescence and anti-endothelial damage of canthaxanthin (CX, which is an active molecule from food) has been studied. **Methods**: This study was performed by adding CX to a model of cell senescence and oxidative damage induced by hydrogen peroxide. Cellular senescence markers (e.g., p16, p21, and p53) and oxidative damage markers (e.g., reactive oxygen species, nitric oxide, malondialdehyde, superoxide dismutase) were evaluated by the enzyme-linked immunosorbent assay, laser scanning confocal microscopy, and Western blotting. **Results**: We found that CX downregulated the expression level of senescence-associated molecules, and significantly reduced the oxidative damage of vascular endothelial cells. These observations showed that CX effectively alleviated the senescence of vascular endothelial cells. Furthermore, CX treatment reduced the expression levels of interleukin-6 (IL-6), tumor necrosis factor alpha, and IL-1β. Finally, *in vivo*, CX significantly alleviated vascular senescence. **Conclusions**: The current study shows that CX has potential application value for treating vascular aging or endothelial cell senescence.

Keywords: Canthaxanthin; senescence; vascular endothelial cells; oxidative damage

1. Introduction

Cardiovascular disease caused by aging has become one of the leading causes of death and disability in the elderly [1]. Aging is inevitable and is a natural physiological process [2]. At present, many countries worldwide are experiencing growth in both the size and proportion of older persons in the population. With increasing age, the structure and function of various organs in the elderly decline, which leads to various age-related diseases such as hypertension, cerebrovascular disease, and diabetes [3]. At present, the morbidity and mortality rates of cardiovascular and cerebrovascular diseases are very high, second only to cancer, with about tens of millions of people dying of cardiovascular-related diseases each year [4]. With the increasing aging population, the rates of cardiovascular disease are increasing rapidly; its complications seriously affect the quality of life of patients, increase their mental burden, and impose a heavy economic burden on society [4,5]. Therefore, the study of aging cardiovascular disease is of great significance. Senescent damage to endothelial cells is one of the drivers of atherosclerotic cardiovascular disease [6]. A large number of epidemiological studies have shown that vascular endothelial cell aging plays a crucial role in the pathogenesis of atherosclerosis, thrombosis and other vascular dysfunction diseases [7–9]. Endothelial dysfunction is an early phenotype of aging-related vascular dysfunction [10]. Delaying vascular aging and preventing cardiovascular disease are major public health issues that urgently need to be addressed [11]. Scientists have studied various drugs to prevent the occurrence and progression of cardiovascular disease, but progress has been very slow.

Canthaxanthin (CX) is a non-vitamin. A source of keto-carotenoid that can be obtained from food and animals [12]. The chemical systematic name (IUPAC) of CX is β, β-carotene-4,4′-dione (molecular formula: $C_{40}H_{52}O_2$), and the relative molecular mass of CX is 564.9 [13]. Studies have shown that CX has preventive and therapeutic effects on a variety of diseases [14], which are related to its biological effects including antioxidant and immune-boosting properties [14,15].

In this study, we established an arterial/venous endothelial cell senescence model to investigate the anti-aging effects of CX. The results show that CX has potential application value for treating vascular aging or endothelial cell senescence.

2. Materials and Methods

2.1 Antibodies

CX was obtained from Shanghai Yuanye Biological Technology Co., Ltd. (S85614-5 mg; Shanghai, China), and dissolved in dimethyl sulfoxide. Primary human aortic endothelial cells (HAECs) were purchased from American Type Culture Collection (ATCC) (Cat No. PCS-100-011; Manassas, VA, USA). Human umbilical vein endothelial cells (HUVECs) were purchased from ATCC. Fetal bovine serum (FBS) (Cat No. 10099141), F-12k medium (Cat No. 21127022), and endothelial cell growth supplement were purchased from Themo Fisher Scientific (Waltham, MA, USA). Trypsin (Cat No. 15090046) and heparin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malondialdehyde (MDA) (Cat No. BC0025) and superoxide dismutase (SOD) (Cat No. S8410) detection kits were purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China). β-actin/p21/p53/B-cell lymphoma 2 (Bcl-2) were from ABclonal Technology (Woburn, MA, USA). Anti-Bcl-2-associated X protein (Bax) and anti-GAPDH were obtained from Proteintech Group (Wuhan, China). Total RNA Extraction Kit, Reverse Transcription Kit, Real-Time Fluorescent PCR Kit, and Lipofectamine 3000 Transfection Reagent were purchased from Bao Bioengineering (Dalian) Co., Ltd. (Dalian, China). Endothelin-1 ELISA kit was purchased from Shanghai Youkewei Biotechnology Co., Ltd. (Shanghai, China). DMEM (Dulbecco's Modified Eagle Medium, Cat No. 12430054) was purchased from Themo Fisher Scientific (Waltham, MA, USA).

2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs, Cat No. PCS-100-010) were cultured in F-12K medium containing 5% FBS, 100 U/mL penicillin (Beyotime, Cat.no C0222, Shanghai, China), and 100 µg/mL streptomycin (Beyotime, Cat.no C0222, Shanghai, China) supplemented with 5 ng/mL, L-glutamine: 10 mM, Heparin sulfate: 0.75 Units/mL, Hydrocortisone: 1 µg/mL, Ascorbic acid (Endothelial Cell Growth Kit-BBE PCS-100-040): 50 µg/mL. Subculturing was performed when the cells had grown to logarithmic phase. Primary human aortic endothelial cel (HAEC) cells were cultured in complete DMEM (Cat No. 12430054) containing 5% FBS supplemented with EGF: 5 ng/mL, L-glutamine: 10 mM, Heparin sulfate: 0.75 Units/mL, Hydrocortisone: 1 µg/mL, Ascorbic acid: 50 µg/mL Primary human aortic endothelial cells were purchased form ATCC (Cat No. PCS-100-011). The above cells have been tested accordingly to exclude mycoplasma contamination. HUVECs and HAEC have been authenticated by STR and sequencing.

2.3 Administration of CX in Vivo

Aging mice (20-month-old female C57BL/6 mice, 25–30 g) were purchased from Beijing Huafukang Company (Beijing, China). The experimental animals were housed in a room with a 12-h day:night cycle and with controlled temperature (22–23 °C). The experiment was divided into two groups (N: 8 mice per group): control group and experimental group. The experimental mice were administered with CX (20 mg/kg, the CX dose used in the current study was chosen based on previous literature reports [16,17]) by intragastric gavage for 8 weeks (5 time/week). At the end of the experiment, blood and organ tissues were collected for further analysis. Animal experiments were approved by the Animal Ethics Committee of Zhongshan Hospital, Fudan University (IACUC-20200326).

2.4 Establishment of an Aging Model of Vascular Endothelial Cells

The cells were divided into different groups according to the requirements of the experiment. Different concentrations of hydrogen peroxide (H_2O_2) were used to establish a cell senescence model. Specifically, cells were treated with H_2O_2 (10–100 µmol/L) for 2 h, after which the cells continued to be cultured for 24 h. The effects of the different H_2O_2 concentrations on cell senescence were evaluated by Sa-β-gal staining as described below.

Sa-β-galactosidase is a marker of cellular senescence; thus, it was used to confirm that the cell senescence model had been successfully established. First, cells were seeded in 6-well plates when the cells reached 80% confluence. After three washes with phosphate-buffered saline (PBS), cells were fixed in paraformaldehyde at room temperature for 5 to 15 min. After washing, cells were incubated with Sa-β-gal staining solution at 37 °C for different time points, and visualized under a microscope.

2.5 Detection of Intracellular Reactive Oxygen Species

After the cells (HUVECs, HAEC) were treated with CX, cells were incubated with the DCFH-DA probe in a CO_2 incubator for 2 h. Then the cells were washed twice with PBS and collected, followed by flow cytometry (BD Biosciences, San Diego, CA, USA) to detect reactive oxygen species (ROS).

2.6 Detection of Nitric Oxide

Nitric oxide (NO) content was detected with the Nitric Oxide Assay Kit (flow cytometry – orange, No. ab219933; Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. In brief, the cells (HUVECs and HAEC) were cultured in 6-well plates. After washing, the cells were stained with NO staining working solution, washed again, and incubated with test solution at 37 °C, followed by flow cytometry to detect NO. The flow cytometer was equipped with adequate filters to measure fluorescence at Ex/Em = 540/590 nm. ELISA kit was used to detect the endothelin-1 content.

2.7 Cell Transfection

HUVECs were seeded and cultured 24 h before transfection. After the cells were cultured to 80% confluence, small interfering RNA (siRNA) targeting Clock1 was transfected into the cells with Lipofectamine 3000 according to the manufacturer's instructions. After 48 h, cells were lysed and proteins were detected by Western blotting.

2.8 Flow Cytometry

Cells were seeded in 6-well plates (5×10^4 cells/well) and cultured for 24 h. The cells were collected, washed twice, and diluted to 1×10^5 cells/mL. Next, 5 µL Annexin V-FITC and 10 µL propidium iodide were added and incubated at room temperature for 20 min in the dark. Then 200 µL binding buffer was added, and the apoptosis rate was analyzed by flow cytometry (BD Biosciences).

2.9 Detection of MDA and SOD Levels

To test the effect of CX on MDA and SOD, the cells (HUVECs and HAEC) were stimulated with CX (5 µM) treatment for 24 h, the cells were then collected. The contents of MDA and SOD in the cells were analyzed using the MDA (Cat No. BC0025) and SOD (Cat No. S8410) detection kits (Beijing Solarbio Technology Co., Ltd.), according to the manufacturer's instructions.

2.10 Western Blot Analysis

HUVECs and HAECs were collected and lysed by sonication at 4 °C, after which the protein was collected. The protein concentration was detected with the BCA assay. The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred to polyvinylidene fluoride (PVDF) membranes, and blocked at room temperature for 2 h. After washing, the membranes were incubated with primary antibodies overnight at 4 °C. After washing the membrane twice, the membranes were incubated with enzyme-labeled secondary antibodies at room temperature for 2 h. β-actin was used as the internal control.

2.11 Hematoxylin and Eosin Staining

Tissues from each group were fixed, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Briefly, tissue sections were incubated with hematoxylin staining solution for 8 min, after which they were rinsed with tap water for 15 min and differentiated with hydrochloric acid in ethanol for 30 s at room temperature. Then the sections were rinsed with tap water and counterstained with eosin for 30 s. After rinsing again with tap water, the samples were dehydrated in gradient alcohol, immersed in xylene to make the tissue transparent, and sealed with neutral gum. Finally, the pathological changes of the aortic endothelial vessels were observed under a light microscope.

2.12 Immunofluorescence Detection

After the cells were treated with CX, the culture medium was discarded. The cells were washed twice with PBS, and fixed in 4% paraformaldehyde at room temperature for 15 min. After washing with PBS three times for 5 min each, the cells were permeabilized with 0.1% Tritonx-100 for 15 min at room temperature. After washing, the cells were blocked in 5% bovine serum albumin (BSA). After incubation for 1 h at 37 °C, primary antibodies were added and incubated at 4 °C. After a 12 h incubation, the cells were treated with Alexa 488-labeled secondary antibody for 60 min. Then the cells were incubated with DAPI for 5 min at room temperature. After washing, the cells were observed under a confocal microscope (FV3000; Olympus, Tokyo, Japan).

2.13 Detection of Mitochondrial Membrane Potential

After the cells were treated with CX, equal volumes of JC-1 staining working solution and cell culture solution were added, and the cells were thoroughly mixed and incubated at 37 °C for 20 min in the dark. During the incubation period, the JC-1 staining buffer (pre-cooled JC-1 staining buffer) was added. After the incubation, the staining working solution was discarded. After washing, the samples were detected by flow cytometry. Under normal conditions, when the intracellular mitochondrial membrane potential (MMP) is high, the JC-1 fluorescent probe aggregates in the mitochondrial matrix to form a polymer (red); whereas when the MMP is decreased, the JC-1 fluorescent probe cannot form a polymer, resulting in green fluorescence.

2.14 Immunohistochemistry

The paraffin sections were soaked twice in xylene (20 min each time) and soaked twice in absolute ethanol, 5 min each time. Then the sections were soaked respectively in 95%, 80%, and 60% ethanol, followed by immersion in a solution containing 3% H_2O_2 for 20 min. After washing, the sections were sealed by incubation in 3% BSA at for 90 min at room temperature. After washing, sections were incubated with primary antibody for 2 h at room temperature. Then the tissue sections were washed again with PBS five times (30 s each), followed by incubation with fluorescently labeled secondary antibody for 30 min at room temperature.

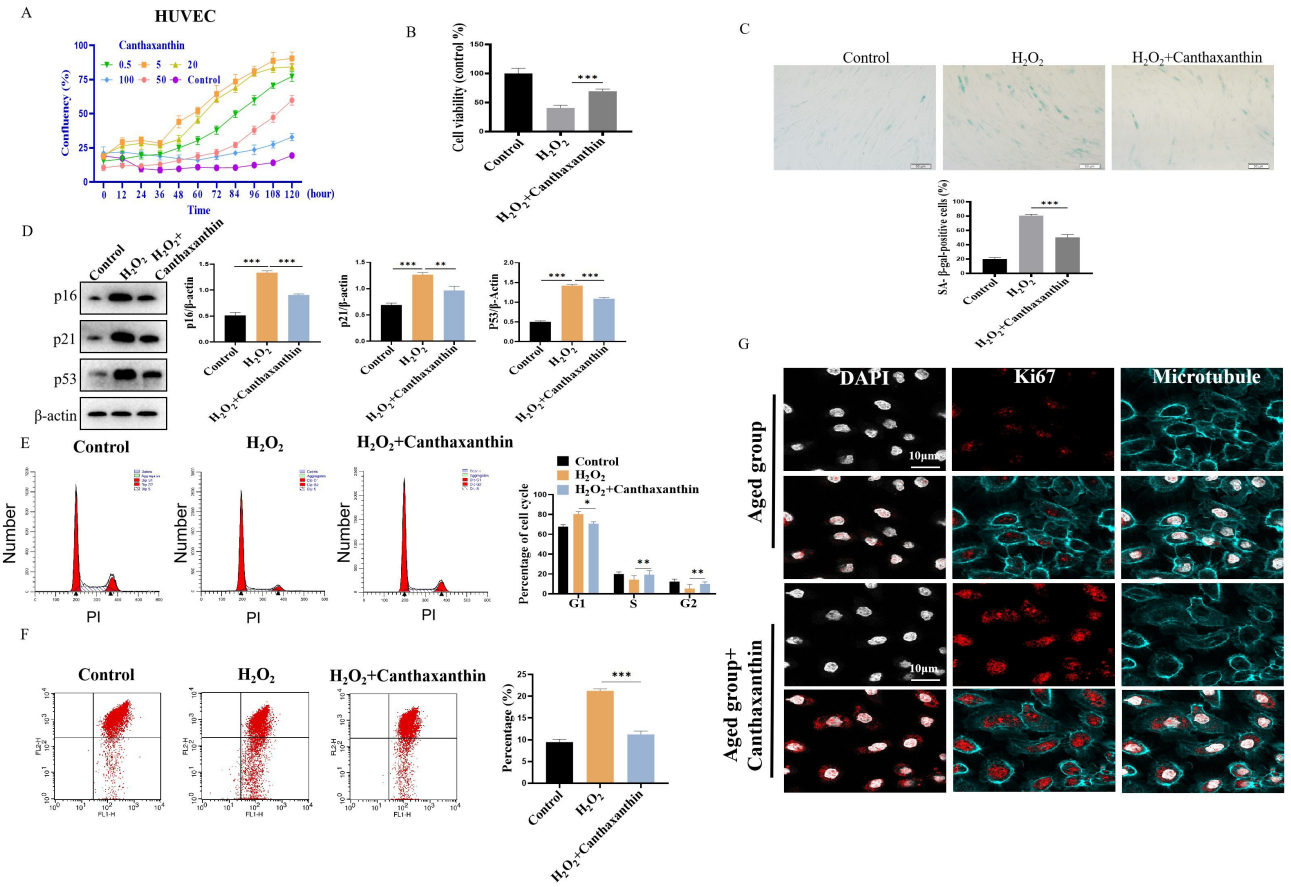

Fig. 1. Effect of canthaxanthin (CX) on cellular senescence. (A) Effects of different concentrations of CX on the human umbilical vein endothelial cells (HUVECs) proliferation. HUVECs were treated with different concentrations of CX for the indicated time points. Cell proliferation was detected by MTT assay. (B) CX enhanced the cell proliferation of the senescent HUVECs. (C) Effect of of CX on SA-β-gal level. (D) CX reduced the p16/p21/p53 expression. (E) Effect of CX on cell cycle. (F) Mitochondrial membrane potential increased in CX intervention group. (G) CX treatment elevated Ki67 expression. n = 3. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

After washing with PBS four to five times (30 s each), the cells were observed under a confocal microscope (FV3000; Olympus).

2.15 Statistical Analyses

Data are expressed as mean values ± standard deviation (SD). Statistical analyses were performed with SPSS19.0 software (IBM Corp., Armonk, NY, USA). Differences between two groups were compared using the Student's *t*-test. Differences between multiple groups were compared using one-way analysis of variance and Tukey's post hoc test. $p < 0.05$ was considered statistically significant. * represent as $p < 0.05$, ** represent as $p < 0.01$ and *** represent as $p < 0.001$.

3. Results

3.1 Effects of CX on the Senescence of Arterial Endothelial Cells

HUVECs were treated with H_2O_2 (10–200 µmol/L) to establish a cell senescence model. According to the results of the pre-experiment, we selected 50 µmol/L H_2O_2 to establish a senescence model of HUVECs (**Supplementary Fig. 1**). Next, to determine the dosage of CX to use in this study, the cells were stimulated with various concentrations of CX (0.5–100 µM). The results showed that CX promoted cell proliferation, which was blocked after treatment with 50 µM CX (Fig. 1A). Therefore, we chose CX at a concentration of 5 µM for subsequent experiments. The MTT assay showed that the cell viability was significantly decreased when the HUVECs were treated with 50 µmol/L H_2O_2 (Fig. 1B); 50 µmol/L H_2O_2 increased the proportion of Sa-β-gal-positive cells (Fig. 1C). Western blot analysis showed that the expression of senescent markers was up-regulated (Fig. 1D). However, in the CX treatment group, the proportion of Sa-β-gal-positive cells was significantly decreased (Fig. 1C), and the expression of p16/p21/p53 was also decreased (Fig. 1D). The number of cells in the S phase of the cell cycle was significantly increased (Fig. 1E), and the MMP was also increased in the CX treatment group (Fig. 1F).

Next, we evaluated the effects of CX on the expression of Ki67. CX treatment significantly increased Ki67 expres-

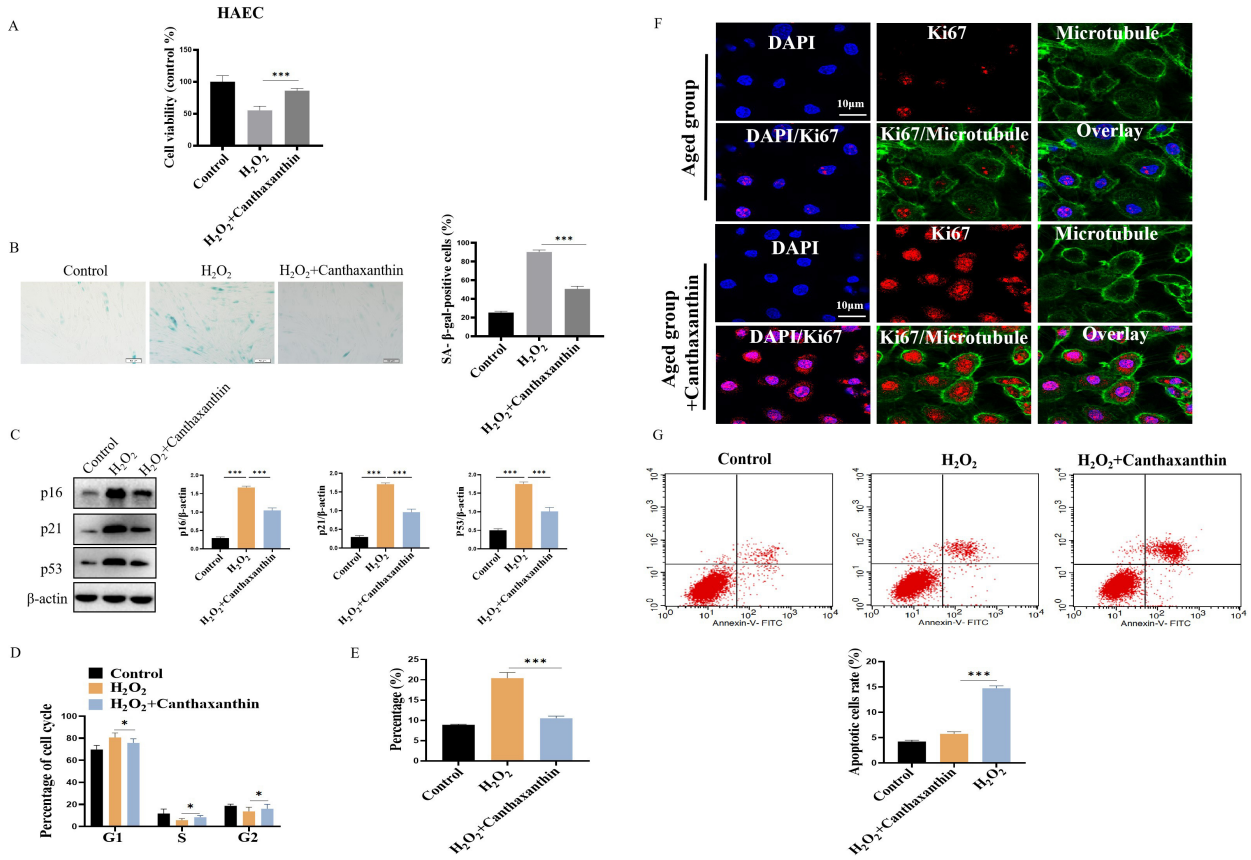

Fig. 2. CX reduced the cellular senescence. (A) CX enhanced the cell proliferation of the senescent human aortic endothelial cells (HAECs). (B) Sa-β-gal positive cells was significantly increased in the CX treatment group compared with the control group. (C) CX treatment down-regulated the expression of senescent markers (p16, p21 and p53). (D) Effect of CX on cell cycle. (E) Mitochondrial membrane potential increased in CX intervention group. (F) CX treatment increased Ki67 expression compared to control group. (G) CX treatment was down-regulated the cell apoptosis rate. n = 3. Data are represented as mean values $\pm$ standard deviation (SD). *$p <$ 0.05 and ***$p < 0.001$.

sion compared with the aging model group (Fig. 1G). Furthermore, CX treatment also downregulated the cell apoptosis rate (**Supplementary Fig. 2**). These results suggest that CX alleviated the senescence of HUVECs.

3.2 Effect of CX on HAEC Senescence

Next, we evaluated the anti-aging effects of CX on HAECs. According to the results of the pre-experiment, we selected 30 μmol/L H_2O_2 to establish the HAEC senescence model. The MTT assay showed that the cell viability was significantly decreased when the HAECs were treated with H_2O_2 (Fig. 2A). Sa-β-gal-positive cells were clearly increased with 30 μmol/L H_2O_2treatment (Fig. 2B). Western blot analysis showed that the expression of senescence markers, p16 and p21, was clearly upregulated (Fig. 2C). However, in the CX treatment group, the cell viability was significantly increased (Fig. 2A), and the number of Sa-β-gal-positive cells was clearly increased (Fig. 2B). Furthermore, p16/p21 expression was downregulated in the CX treatment group (Fig. 2C). Flow cytometry analysis showed that the number of cells in the S phase of the cell cycle was also significantly increased (Fig. 2D), and the MMP was also increased in the CX treatment group (Fig. 2E). Next, we evaluated the effect of CX on the expression of Ki67. The results showed that compared with the senescence model group, the CX treatment significantly increased Ki67 expression (Fig. 2F). Furthermore, CX treatment also significantly reduced the cell apoptosis rate (Fig. 2G).

3.3 Effects of CX on Inflammation of HUVECs and HAECs

We found that CX treatment reduced the levels of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 in HUVECs and HAECs (Fig. 3A). Additionally, CX reduced the expression of inflammatory-related molecule (nuclear factor kappa B) (Fig. 3B), which may be one of the potential molecular mechanisms by which CX relieves inflammation.

3.4 Analysis of the Potential Mechanism by Which CX Exhibits Anti-Aging Effects

A recent study showed that Clock1 is involved in the aging process of stem cells [17]. We found that the expression of Clock1 was significantly downregulated in senes-

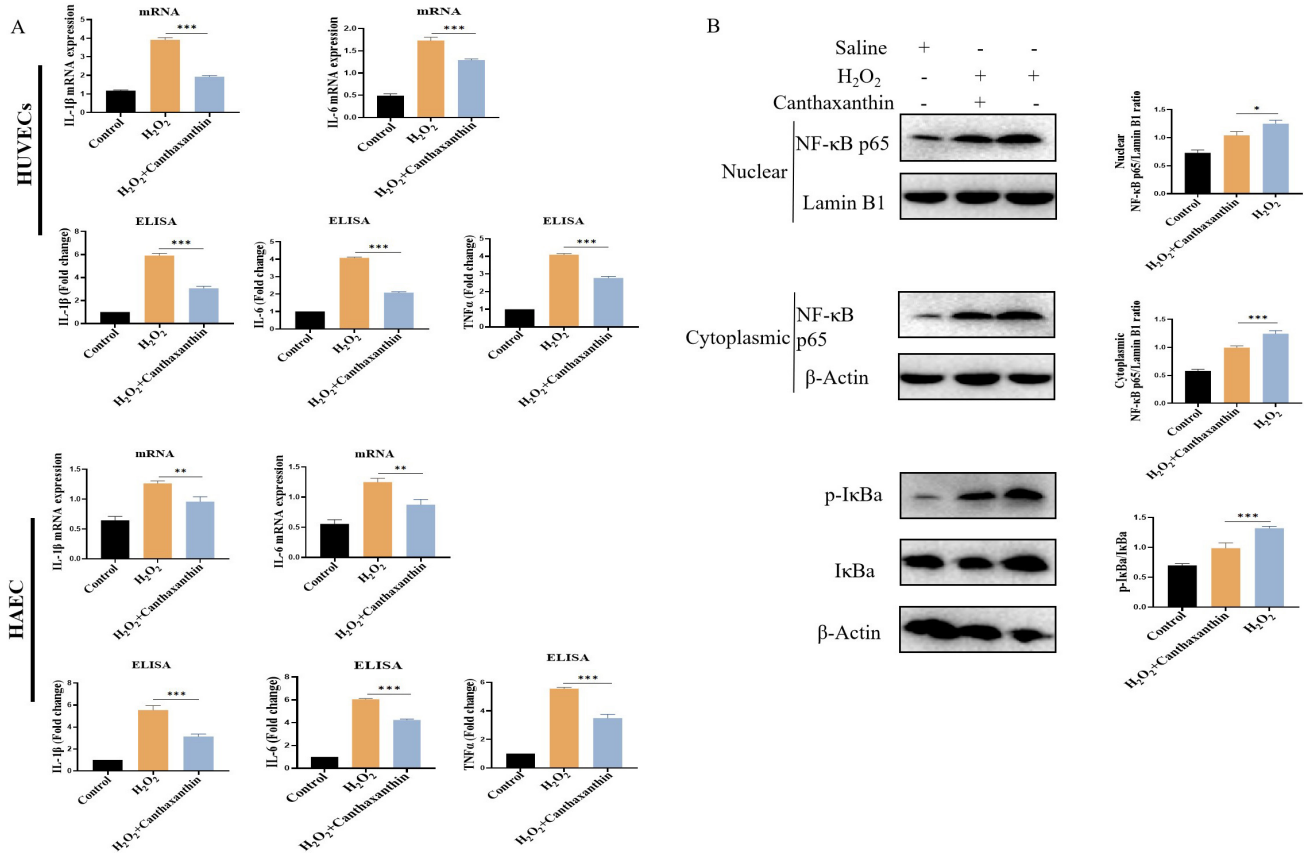

Fig. 3. Effect of CX on cell senescence. (A) CX treatment reduced the levels of the tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and IL-6 in HUVECs and HAEC cells. (B) CX treatment reduced the expression of inflammatory-related molecule (nuclear factor kappa B (NF-κB)). Data are represented as mean values $\pm$ standard deviation (SD). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

cent cells induced by H_2O_2 (Fig. 4A), whereas Clock1 expression was increased after CX treatment (Fig. 4B). Therefore, we speculated that CX exerts its anti-aging effects by regulating Clock1 expression. To test this hypothesis, we treated the vascular endothelial cells with CX, and at the same time, downregulated the expression of Clock1 with siRNA. The results showed that Clock1 knockdown reversed the anti-aging effects of CX, as determined by evaluation of the corresponding senescence markers (Fig. 4B). These findings suggest that CX reduces (at least in part) the aging of the vascular endothelial cell senescence via upregulation of Clock1 expression.

3.5 Effects of CX on Intracellular NO and Endothelin-1

NO and endothelin-1 (ET-1) are markers of endothelial cell damage. When vascular endothelial cells are damaged, NO is downregulated and ET-1 is upregulated [18]. Here, we analyzed whether CX may improve endothelial cell function. The results showed that with CX treatment, the level of NO was significantly increased and the level of ET-1 was significantly decreased compared to the H_2O_2 group (Fig. 5A). Indirect immunofluorescence assay (IFA) showed similar results (Fig. 5B).

3.6 Effects of CX on DNA Double-Strand Damage in the Vascular Endothelial Cells

To further evaluate the effects of CX on endothelial cell damage, we assessed the protective effects of CX on the DNA damage of HUVECs. DNA damage response is one of the consequences of oxidative damage. Gamma-H2AX is considered to be one of the markers of DNA double-strand damage, which is involved in the process of cellular oxidative damage induced by various factors. Western blot analysis showed that the expression level of gamma-H2AX in the H_2O_2 treatment group was significantly higher than that of the control group. Compared with the H_2O_2 group, after CX treatment, the gamma-H2AX expression was significantly reduced (Fig. 6A). In addition, indirect immunofluorescence experiments confirmed this finding (Fig. 6B).

3.7 Effects of CX on the Oxidative Damage of Vascular Endothelial Cells

We assessed the effects of CX on oxidative damage using two cell models, HUVECs and HAECs. The results showed that intracellular ROS was significantly decreased with CX treatment ($p < 0.05$; Fig. 7A). Additionally, we also evaluated the effects of CX on MDA and SOD. Treat-

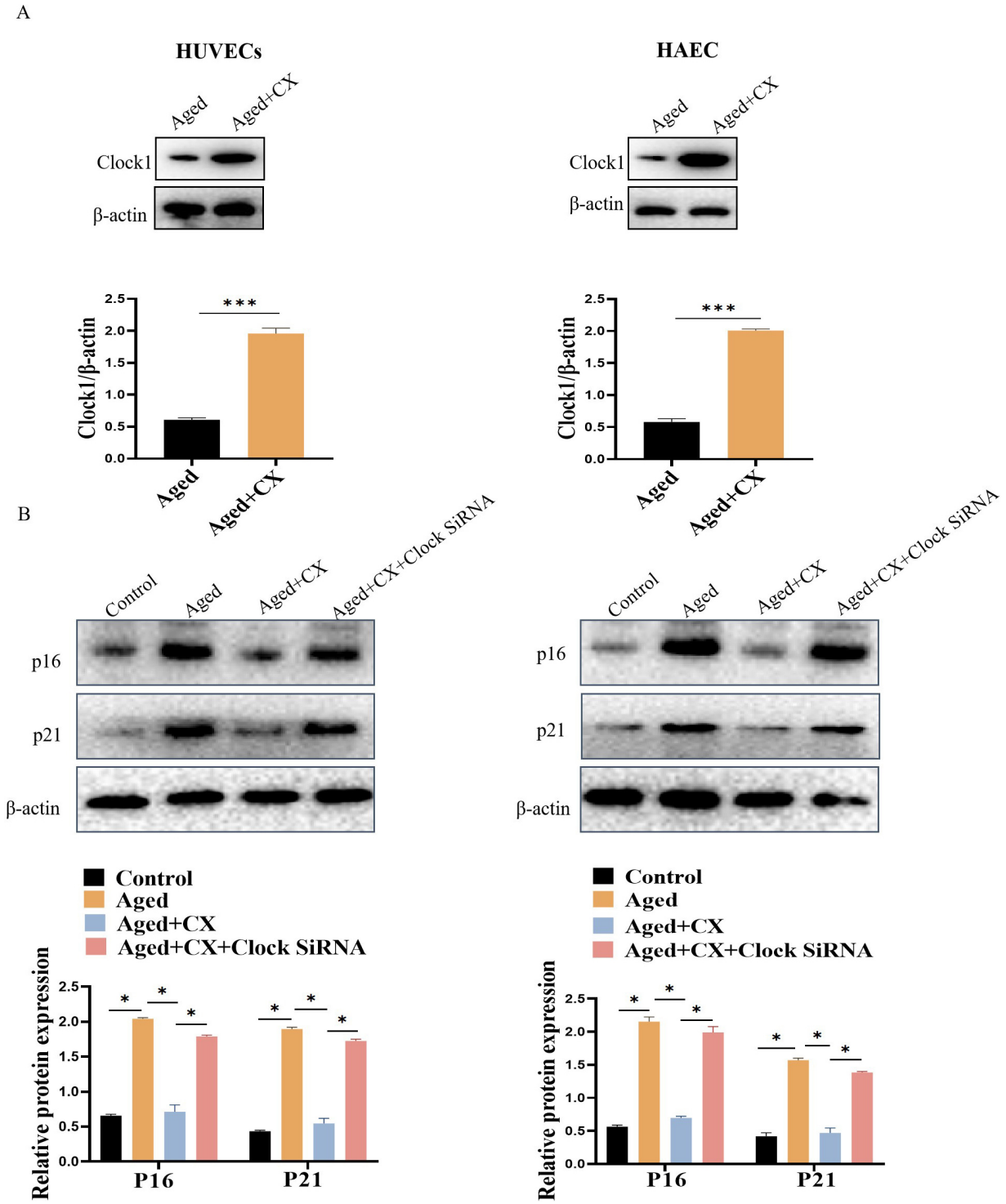

Fig. 4. Effect of CX on Clock1 expression. (A) The expression of Clock1 was significantly down-regulated in the senescent cells induced by hydrogen peroxide (H_2O_2)(left panel: HUVECs; right panel: HAECs). (B) CX relieved the aging by regulation of Clock1 (left panel: HUVECs; right panel: HAECs). Data are represented as mean values ± standard deviation (SD). *$p < 0.05$ and ***$p < 0.001$.

ment with 5 μM CX led to a significant increase in intracellular SOD, and a significant decrease in MDA level (Fig. 7B; $p < 0.05$) (Fig. 7B).

3.8 Analyses of Anti-Vascular Aging Effects of CX in Vivo

We evaluated the potential effects of CX on the blood vessels *in vivo*. We chose a dose of 20 mg/kg to treat 20-month-old mice by oral gavage for 8 weeks. We collected the blood vessels for subsequent analyses, and found that

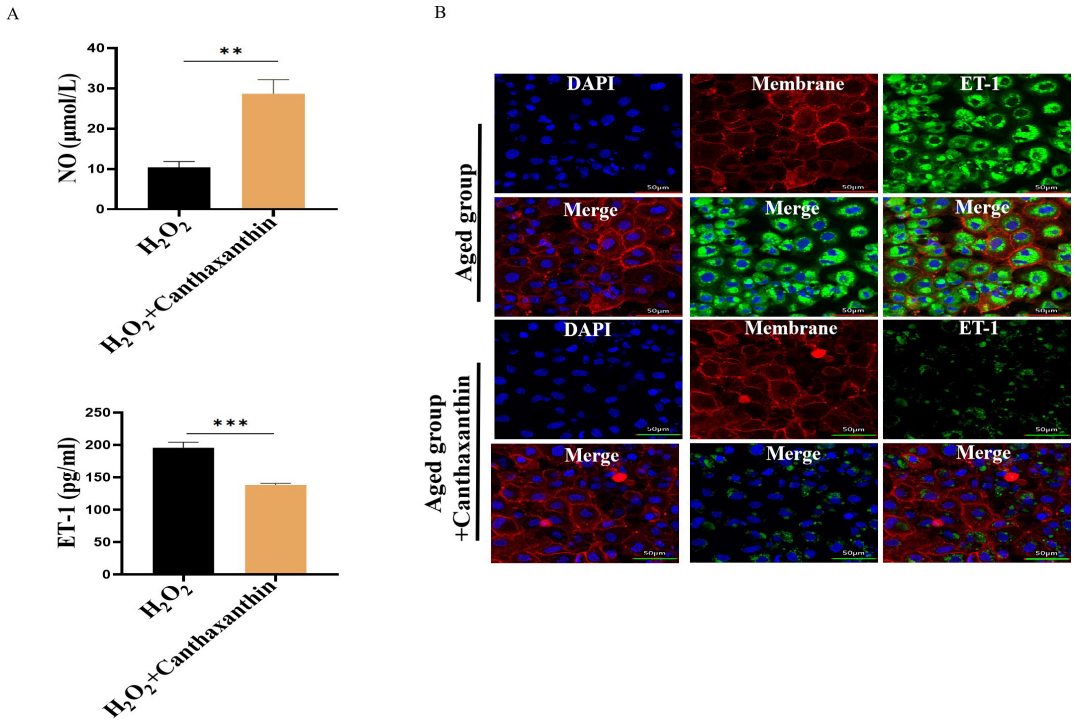

Fig. 5. Effect of CX on NO level and ET-1 expression. (A) CX treatment increased the NO level and decreased the level compared to H_2O_2 group. (B) Endothelin-1 (ET-1) expression was down-regulated by Indirect immunofluorescence assay (IFA) analysis (n = 5). Data are represented as mean values ± standard deviation (SD). $^{**}p < 0.01$ and $^{***}p < 0.001$.

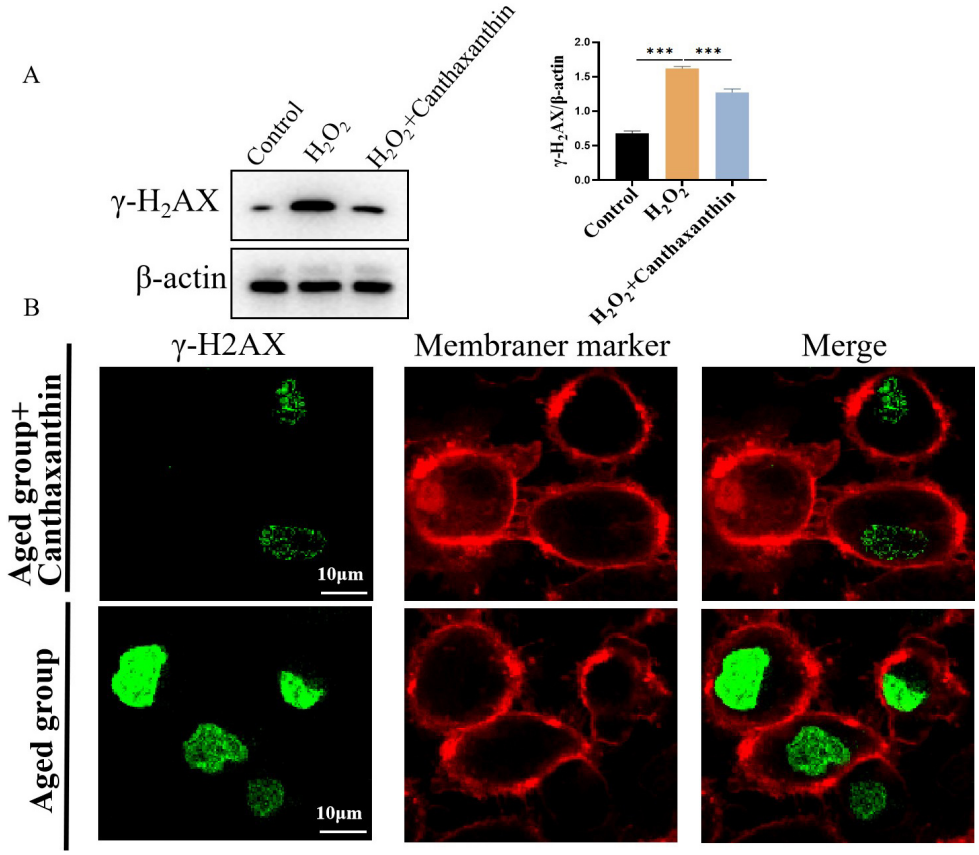

Fig. 6. Effect of CX on γ-H2AX expression. (A) CX treatment reduced the expression of γ-H2AX. (B) γ-H2AX expression was declined in the CX group (n = 3). Data are represented as mean values ± standard deviation (SD). $^{***}p < 0.001$.

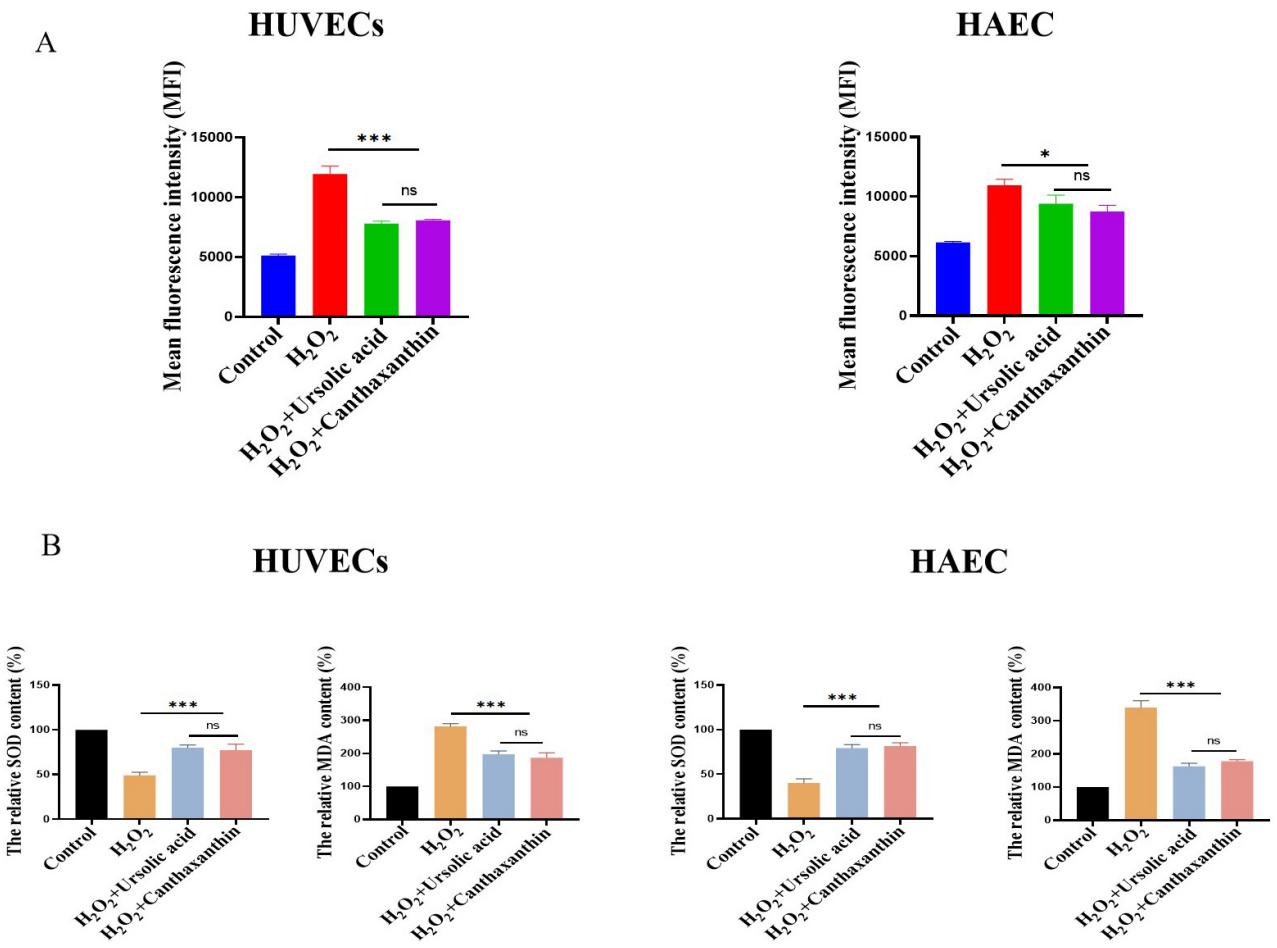

Fig. 7. The effect of CX on oxidative damage of HUVECs and HAEC. (A) The fluorescence intensity of the intracellular reactive oxygen species (ROS) was decreased under the intervention of CX. (B) CX and Ursolic acid treatment enhanced the intracellular superoxide dismutase (SOD) level, and decreased malondialdehyde (MDA) level (n = 5). Data are represented as mean values ± standard deviation (SD). ns, not significant. *$p < 0.05$ and ***$p < 0.001$.

the aging of blood vessels was obvious, which was alleviated by treatment with CX. The levels of p16 and p21 were reduced in the CX treatment group (Fig. 8A). These observations indicated that CX exhibited potential anti-aging effects. Furthermore, we also detected the expression of Ki67 and found that the Ki67 was increased in the CX treatment group (Fig. 8B). In addition, endothelin-1 expression was also reduced (Fig. 8C), suggesting that vascular damage was reduced after CX treatment. The above findings were also confirmed by western-blot analysis (**Supplementary Fig. 3**).

4. Discussion

Many countries in the world are moving towards an aging society. The aging of the population brings serious consequences to the society [19–23]. To date, pharmacological interventions to reverse age-related endothelial dysfunction have had limited effect. Therefore, it is necessary to find new bioactive molecules to treat or alleviate the aging damage of cardiovascular cells. For this, we established the arterial/venous endothelial cell senescence model to investigate the anti-aging effects of CX. The results indicated that CX exhibit good anti-aging potential *in vivo* and *in vitro*.

Vascular endothelial cell senescence is one of the major risk factors for vascular aging and cardiovascular disease [24]. Vascular endothelium is located in the innermost layer of blood vessels and is the barrier between blood and blood vessels. Vascular endothelial cells can synthesize and release a variety of active substances to maintain vascular homeostasis [25]. Vascular endothelial dysfunction is the earliest pathological change in atherosclerosis. Human umbilical vein endothelial cells (HUVECs) are derived from the venous endothelium of human umbilical cord, which is relatively easy to obtain, and it is also one of the most commonly used vascular endothelial cells *in vitro*, it can be used to study the function of vascular endothelial cells. Therefore, we have successfully established a senescence model of venous endothelial cells using H_2O_2. On this basis, we also established a senescence model of arterial endothelial cells. Further experimental observations indicated that CX mitigates the senescence of vascular endothelial

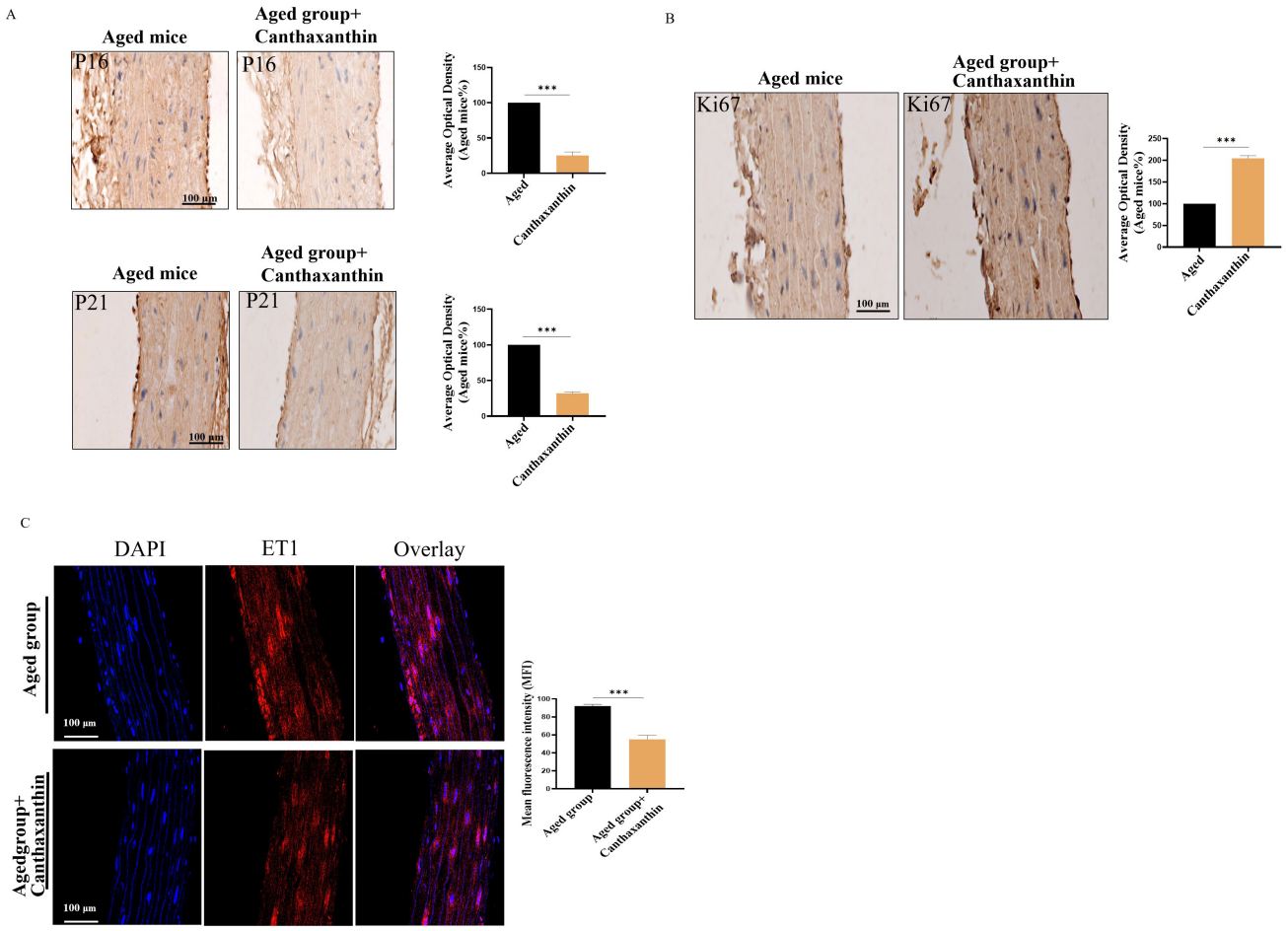

Fig. 8. Effect of CX on aging of vascular tissue. (A) The expression of p16 and p21 was down-regulated in CX treatment *in vivo*. (B) The Ki67 expression was increased. (C) ET-1 expression was reduced (n = 5). Data are represented as mean values ± standard deviation (SD). ***$p < 0.001$.

cells. In addition, Western-blot analysis also showed that aging-related marker molecules were significantly down-regulated (such as P16 and P21). These findings suggest that CX can effectively alleviate the senescence of vascular endothelial cells.

Aging is closely related to the increase of cellular inflammatory factors and chronic low-level inflammation. Therefore, we further studied the effects of CX on inflammatory factors in senescent cells, including interleukin-6, TNF-α and IL-1β. We found that CX down-regulates the expression level of IL-6, TNF-α and IL-1β, indicating that CX could alleviate the inflammation of arterial and venous cells.

A series of documents have reported various physiological functions of carotenoids (including β-carotene and lutein), such as antioxidant, immune promotion and enhancement of intercellular gap junction communication [14]. CX, as a carotenoid, has been reported to have many biological functions [26]. CX can improve the immunity in animals [27]. In addition, CX has anti-cancer effects [28]. But in this work, we found for the first time that CX has an anti-vascular aging effect. Compared with other drugs, CX has several advantages. Anti-aging is a long-term process. CX is edible and exists in some vegetables. Secondly, CX can be used as a health food, because β-Carotene-4,4′-dione itself is a biologically active molecule that exists in natural plants, so it is green and safe. It can be used for a long time.

Oxidative stress is one of the main mechanisms of cellular senescence [29]. When cells are under oxidative stress, excessive ROS is generated, a large amount of free radicals are generated. Cells are damaged, mitochondrial DNA is damaged. In the current study, the experimental results *in vitro* and *in vivo* show that CX could significantly alleviate endothelial cell damage by detecting SOD, MDA and other related markers [30], but the specific molecular mechanism still needs in-depth research in the future. In addition, the level of NO was significantly increased, and the level of ET-1 was significantly down-regulated, indicating that CX could alleviate the damage of vascular endothelial cells.

In the current study, we have to ask how does CX mitigate cellular senescence? This is a very interesting but at the same time difficult question to answer. This is because

CX could internalize into cells. After internalization, CX exerts its anti-aging effects though interacting with intracellular molecules. However, the specific molecules with which it interacts to produce its anti-aging effects are not fully understood. Therefore, we think that CX may exert its anti-aging effects in multiple ways, because (1) there are numerous factors that contribute to aging, such as inflammation, oxidative stress, mitochondrial destabilization, and genomic instability. In the current study, we found that CX could alleviate inflammation and oxidative stress, which may be one of the mechanisms for its anti-aging effects, but may not be the only one; (2) we also found that CX can up-regulate Clock1 by which CX exerts its anti-aging effects, because the expression of Clock1 is significantly down-regulated in senescent vascular cells, Clock had been shown to be closely associated with aging [17]; (3) we believe that mitochondria are one of the targets of CX action because mitochondria is important organelle for ROS production. In the current study, the ability of CX to alleviate oxidative stress may be related to mitochondria. Mitochondria is an important anti-aging target. The recent outstanding literature reviewed that nutraceuticals and dietary supplements may alleviate aging and oxidative stress by modulating mitochondria [31,32]. These findings strongly suggest that CX may exert its anti-aging effects through multiple pathways. Of course, the current study cannot exclude that CX may also exert its anti-aging effects through other unknown molecular pathways. These scientific questions need to be addressed in future studies, which requires the combined efforts of many scientists.

Of course, there are still some limitations in this study. (1) Firstly, the molecular mechanism of CX exhibiting its anti-aging needs to be studied in depth, because the current study did not identify the direct target of CX by which CX displays its anti-aging effect; (2) Secondly, further *in vivo* experiments are needed. We have only preliminarily investigated the anti-aging effects of CX *in vivo* in this work, so further studies are needed for in-depth evaluation of the anti-aging effects of CX *in vivo*.

Scientists have found that CX has antioxidant effects, which may be the core mechanism by which CX exhibits anti-aging effects. In addition, we further explored whether there are other potential mechanisms by which CX exhibit anti-aging effects. After screening, we found that CLOCK may also be involved in the anti-aging process of CX, because we observe that CX exhibits anti-aging effect by regulating CLOCK via a rescue experiment. Of course, this is only a preliminary result, which need systematic and deep research in the future.

5. Conclusions

In the current study, we investigated the effect of CX on the aging damage. We firstly found that CX attenuates the senescence of vascular endothelial cells. Further research showed that CX suppresses the oxidative stress and inflammation. Mechanistic studies suggest that CX exhibits its anti-aging effects possibly through the regulation of Clock1 expression. In addition, we also found that CX suppresses the aging of blood vessels *in vivo*. Taken together, we found that CX could mitigate the aging and oxidative damage of vascular endothelium cells, indicating that CX has good potential for anti-aging applications.

Abbreviations

CX, Canthaxanthin; FBS, Fetal bovine serum; SOD, superoxide dismutase; ROS, reactive oxygen species.

Availability of Data and Materials

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

Author Contributions

ZFW, KDZ and LJC designed the research study; LLL, SQL performed the research; YBJ provided help and advice on the conclusions; RH, JF analyzed the data; ZFW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Animal experiments were approved by the Animal Ethics Committee of Zhongshan Hospital, Fudan University (IACUC-20200326).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2812367.

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