SPANDIDOS PUBLICATIONS

Anti-oxidation effect of Genistein in vascular endothelial cell after H_2O_2 stress

KUN XU[1], QINGWU QIN[2], YE YAO[3], LIN YUAN[3], XIZI DU[3], KAI ZHOU[3],
XINYU WU[3], WEIJIE WANG[3] and CHI LIU[3]

[1]School of Medicine, Hunan Normal University, Changsha, Hunan 410013; [2]Department of Pulmonary and Critical Care Medicine, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011; [3]Department of Physiology, Xiangya School of Medicine, Central South University at Changsha, Changsha, Hunan 410013, P.R. China

Received April 21, 2023; Accepted September 20, 2023

DOI: 10.3892/mmr.2023.13125

Abstract. Atherosclerosis (AS) is a chronic inflammatory disease characterized by increased oxidative injury in vascular endothelial cells. Inhibiting the oxidative damage of vascular endothelial cells can effectively prevent the occurrence and development of AS. Of note, Genistein (GEN; ID no. 5280961) is phytochemical found in legume family which has flavonoid properties with multiple potential biological activities including antioxidant, anti-inflammatory and anticancer. Antioxidant capacity of GEN has a potential protective effect on vascular endothelial cells after oxidative stress. In the present study, the protective effect of GEN on H_2O_2-induced oxidation damage was investigated in human vascular endothelial cells (HUVECs). Following GEN pretreatment of HUVECs, H_2O_2 was added, and apoptosis was detected by flow cytometry, and the expression of relevant genes and proteins was detected by PCR and westerner blot. The results of the present study revealed that GEN significantly enhanced the cell survival rate and decreased the apoptotic rates of HUVECs after H_2O_2 stress. Besides, GEN reduced the accumulation of intracellular reactive oxygen species by enhancing activity of antioxidant enzymes glutathione peroxidase, superoxide dismutase (SOD) and glutathione peroxidase. Moreover, GEN also inhibited the apoptosis of vascular endothelial cells and enhanced the activation of the nuclear factor erythroid2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1)/SOD pathway. Collectively, it was identified that GEN is an effective antioxidant which can reduce the oxidative damage by H_2O_2 through the Nrf2/HO-1/SOD signaling pathway in HUVECs.

Correspondence to: Professor Chi Liu, Department of Physiology, Xiangya School of Medicine, Central South University at Changsha, 172 Tongzipo Road, Changsha, Hunan 410013, P.R. China
E-mail: liu.chi@csu.edu.cn

Key words: genistein, anti-oxidation effect, vascular endothelial cell, oxidative damage

Introduction

A total of ~20 million people worldwide succumb every year due to AS with the increased of the incidence rate of atherosclerosis (AS) (1). In 2018, the death induced by cardiovascular disease was the first cause of death among urban and rural residents in China (2). Of note, vascular endothelial cells are lined up in the inner surface of blood vessels, are in direct contact with the metabolite-related endogenous danger signals in the circulatory system. Consequently, the impairment and dysfunction of vascular endothelial cells would impair vasodilation and increase endothelium-dependent permeability, which is strongly correlated with the development of AS. Reactive oxygen species (ROS) is the key factor that contributes to the injury of vascular endothelial cells (3). Therefore, protecting endothelial cells from the damage of ROS is one of the effective strategies to prevent AS. As H_2O_2 is a well-known ROS, it is often used as a stimulant of *in vitro* model for oxidative injury in AS which presented as ROS-intermediated destruction of lipids and proteins contributing to damage of the membrane in a series of cells (4).

It is known that soy is the main source of high-quality proteins (5) and the nutritional value of soy is mainly attributed to the content of phytochemical content, particularly the uniquely rich content of isoflavone (6). Particularly, Genistein (GEN), the most active soy isoflavone, is a potent antioxidant and anti-browning agent both *in vivo* and *in vitro*, which exhibit preventive and therapeutic effects on cancer, post-menopausal syndrome, osteoporosis and a series of cardiovascular diseases (7,8). GEN pretreatment significantly attenuated H_2O_2-induced peroxide formation and inhibited ischemia-induced ROS production, including enhancement of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in Caco-2 cells and cerebral ischemia mouse (9,10). Moreover, GEN can also attenuate apoptosis by reversing the ratio of Bcl-2 to Bax and inhibiting the activity of the pro-apoptotic caspase-9 and caspase-3 in the primary rat neurons (11).

However, the effect of GEN on endothelial cells after oxidative damage remains obscure and the mechanism by which GEN attenuates oxidative stress-induced endothelial cell injury also needs further study to verify. Therefore, the

present study aimed to further determine the effect of GEN on the oxidative damage of human vascular endothelial cells (HUVECs) caused by H_2O_2 and further elucidated its potential associated signaling pathways.

Materials and methods

Cell culture. HUVECs were provided by the Basic Medical College of Jilin University (Changchun, China). In brief, HUVECs were cultured in RPMI-1640 medium (P004-1, Nanjing Jiancheng Bioengineering Institute) containing 10% fetal bovine serum (F8318, Gibco) and 1% penicillin in a 37˚C, 5% CO_2 incubator. The treatment conditions of H_2O_2 and GEN (cat. no. HY-14596; MedChemExpress) were determined according to previous studies (10,12). Subsequent experiments were divided into four groups: control group, GEN treatment group, H_2O_2 treatment group and a combined treatment group of GEN and H_2O_2.

Cell viability assay. H_2O_2 (130 μM) was used to induce oxidation injury in HUVECs (1x10^5 cells per well was seeded) according to pre-experiments and previous studies (10,12). GEN with different concentrations (100 nM, 1 μM, 10 μM) were pretreated to reduce the oxidant injury of H_2O_2. The CCK-8 kit (ck04, Dojindo, the absorbance values at 450 nm were recorded) was used to detect cell viability referring to operating instructions. A total of 10 μl CCK-8 solution was added to each well and incubated at 37˚C with 5% CO_2 in incubator for 2 h. An optimal dose of GEN was utilized for its antioxidant effect and for observing its antioxidant mechanism.

Intracellular ROS assay. HUVECs were seeded in six-well plates at a density of 1.2x10^5/ml per well for 24 h. Then, Dichloro-dihydro-fluorescein diacetate (DCFH-DA) Assay (cat. no. S0033S; Beyotime Institute of Biotechnology) was implemented. A total of 10 μmol/l GEN was added to the GEN and GEN + H_2O_2 group for 1 h; 130 μmol/l H_2O_2 was added to the GEN + H_2O_2 group and the H_2O_2 group for 1 h. After the treatment was completed, the six-well plates were taken out from the incubator and washed by adding 1 ml of PBS to each well once, then the PBS was discarded. The DCFH-DA probes were diluted with the serum-free RPMI-1640 culture medium at the ratio of 1:1,000. The discarded culture medium from the six-well plates was aspirated, and the cells were washed three times with 1 ml of serum-free RPMI-1640 culture medium per well to avoid eluting the cells from the wall. After aspirating the culture medium used for washing, 1 ml of prepared DCFH-DA probe was added to each well and the six-well plate loaded with the probe was incubated in the incubator for 40 min to prevent degradation and inactivation of the probe, and the HUVECs were washed with serum-free cell RPMI-1640 culture medium for 3 times after 40 min to wash away the DCFH-DA that had not entered into the cells. Then, 200 μl of 0.25% trypsin was added to each well, the digestion was terminated, centrifugation followed and the supernatant was discarded. A total of 200 μl of PBS was added to each tube and was blown several times to make a single-cell suspension, which was transferred to a flow tube protected from light, and put on a flow cytometer (C6, BD, USA, 488 nm excitation light and 525 nm emission used) for ROS detection.

Apoptosis assay. Apoptosis was detected using Annexin V-FITC kit (cat. no. C1062L; Beyotime Institute of Biotechnology) (13). In brief, HUVECs were seeded in six-well plates at a density of 1.2x10^5/ml per well for 24 h. Then, the desired concentrations of GEN and H_2O_2 were added and washed once with PBS according to previous description. After that, cells were collected and supplemented with Annexin V-FITC conjugate. The flow tubes were incubated for 20 min in a dark place at room temperature and analyzed immediately by a flow cytometer (C6 and BD Accuri™ C6 Software, version 227.4).

Determination of intracellular enzyme activity. Cells in logarithmic growth phase were cultured in 5-mm cell culture dishes, and cell samples were collected after adding GEN and H_2O_2. The cells were removed from the incubator, placed in an ice bath, and the adherent cells were collected in 1.5 ml EP tubes by scraping with a cell scraper. A total of 100 μl of cell lysate was added to each tube and placed in liquid nitrogen for 3 min, 37˚C water bath for 3 min, and repeated three times. The cells were pre-cooled at 4˚C in advance in a centrifuge and centrifuged at 12,000 x g for 10 min, and the supernatant was taken for the assay of enzyme activity. The intracellular enzyme activity was detected according to the instructions of GPx kits (cat. no. S0058; Beyotime Institute of Biotechnology) (14), SOD kits (cat. no. BC0175; Beijing Solarbio Science & Technology Co., Ltd.) (15), and reduced glutathione (GSH) kits (cat. no. BC1175; Beijing Solarbio Science & Technology Co., Ltd.), respectively (16).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. RNA [An RNA extraction kit was used (12183018A, PureLink™ RNA; Thermo Fisher)] was extracted from HUVECs and qPCR [The TBGreen® Premix Ex Taq (Takara) was used] was conducted after reverse transcription according to the reagent manufacturer's instructions (T2210, Solarbio, China). GAPDH was used as a reference gene. Primer sequences are listed in Table I. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles including denaturation at 95˚C for 10 sec, annealing at 55˚C and extension at 72˚C for 30 sec. A standard measure of mRNA expression (2-ΔΔ cycle threshold) was calculated (17).

Western blot analysis. A total of 50 μg of cell protein were collected from four groups [Negative control (NC), GEN, GEN + H_2O_2, H_2O_2] with a cell scraper and lysed with RIPA (cat. no. P0013B; Beyotime Institute of Biotechnology) and 1% PMSF (cat. no. ST506; Beyotime Institute of Biotechnology). Electrophoresis was carried out, placing 50 μg of protein in each well, using 10% preformed gel. After transferring the proteins to the membrane (PVDF), it was blocked with 5% skimmed milk powder at room temperature for 2 h. Subsequently, the membrane was incubated overnight at 4˚C with the following primary antibodies (all diluted at 1:1,000): Nuclear factor erythroid2-related factor 2 (Nrf2; cat. no. 12721S), heme oxygenase (HO-1; cat. no. 43966), SOD1 (37385S), Bax (cat. no. 5023S; all from Cell Signaling

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene name	Primer sequences (5'→3')
Nuclear factor erythroid 2-related factor 2	F: AGTCCAGAAGCCAAACTGACAGAAG R: GGAGAGGATGCTGCTGAAGGAATC
Superoxide dismutase 1	F: ATCCTCTATCCAGAAAACACGG R: GCGTTTCCTGTCTTTGTACTTT
Heme oxygenase-1	F: CCTCCCTGTACCACATCTATGT R: GCTCTTCTGGGAAGTAGACAG
Bax	F: CGAACTGGACAGTAACATGGAG R: CAGTTTGCTGGCAAAGTAGAAA
Bcl-2	F: GACTTCGCCGAGATGTCCAG R: GAACTCAAAGAAGGCCACAATC
Caspase-3	F: CCAAAGATCATACATGGAAGCG R: CTGAATGTTTCCCTGAGGTTTG
GAPDH	F: AGATCCCTCCAAAATCAAGTGG R: GGCAGAGATGATGACCCTTTT

F, forward; R, reverse.

Technology, Inc.), Caspase-3 (cat. no. 14220S; Abcam) and Bcl-2 (cat. no. orb10173; Biorbyt, Ltd). Following the primary incubation, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; cat. no. 7074S; Cell Signaling Technology, Inc.) for 1 h at room temperature. As the same grouping (NC, GEN, GEN + H_2O_2, H_2O_2) in western blot detection, the same GAPDH (cat. no. AC001; ABclonal Biotech Co., Ltd.) was used as control in each experiment which was repeated at least three times. ImageJ software (1.52a, National Institutes of Health) was used to analyze the grey value of each resulting image, and the control protein was used to normalize the grey values of the target protein for statistical analysis.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 (Dotmatics). One way ANOVA was used for multiple group comparisons, corrected by Bonferroni's method. Two independent samples unpaired t-test was used for comparison between two groups. All experiments were repeated more than 3 times. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of GEN on the viability of HUVECs after H_2O_2 stress. To determine whether GEN could play a protective role against H_2O_2-induced cell damage, in combination with H_2O_2 stress for 1 h, HUVECs were pretreated with GEN (100 nM, 1 and 10 μM) for 1 h. As demonstrated in Fig. 1, the cell viability increased significantly after pretreatment of GEN compared with control group (****P<0.0001). Thus, 10 μM GEN pretreatment for 1 h was used as the treatment condition for the following experiments.

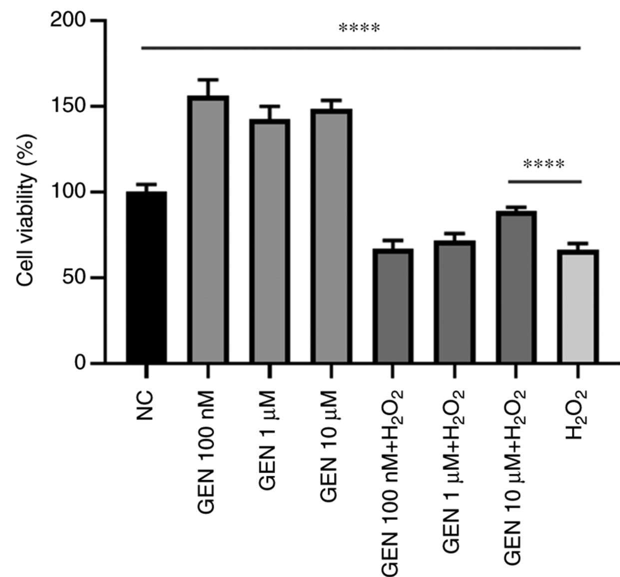

Figure 1. Effect of GEN pretreatment on cell viability after H_2O_2 stress. The cell viability increased significantly after pretreatment of GEN compared with the control group. ****P<0.0001. GEN, Genistein.

Effect of GEN on oxidative injury-induced intracellular ROS. To investigate whether GEN could reduce the level of intracellular ROS, DCFH-DA fluorescent probe was used to detect the intracellular ROS in the labeled cells. As revealed in Fig. 2, intracellular ROS levels increased significantly after H_2O_2 stress (*P<0.05). By contrast, after GEN pretreatment, the intracellular ROS levels reduced significantly (*P<0.05).

Effect of GEN on the apoptosis of HUVECs after H_2O_2 stress. To verify whether GEN could further attenuate the occurrence of apoptosis by attenuating the aggregation of ROS, Annexin-V/FITC Assay was applied to stain cells pretreated with the GEN. The apoptotic rates were counted separately for different periods. As shown in Fig. 3, GEN decreased the early apoptotic rate and the total apoptotic rate significantly (P<0.05), while no effect was detected for the late apoptotic rate.

Effect of GEN on intracellular enzyme activity of HUVECs after H_2O_2 stress. To further verify the mitigating effect of GEN on the intracellular ROS, intracellular redox-responsive enzymes were measured. As demonstrated in Fig. 4A, the enzyme activity of intracellular GPx was significantly reduced in the H_2O_2 group (*P<0.05) and increased after GEN pretreatment (*P<0.05). The intracellular SOD activity was also significantly reduced (**P<0.01) and increased after GEN pretreatment (*P<0.05; Fig. 4B). In addition, the activity of intracellular GSH was significantly reduced in the H_2O_2 group (***P<0.001) and increased after GEN pretreatment (***P<0.001; Fig. 4C).

Effect of GEN on the expression of Nrf2-related signaling pathway. To verify whether the protective effect of GEN on oxidative damage after H_2O_2 stress was mediated through the Nrf2 pathway, the expression of Nrf2 pathway-related molecules (Nrf2, HO-1 and SOD1) was detected. As revealed

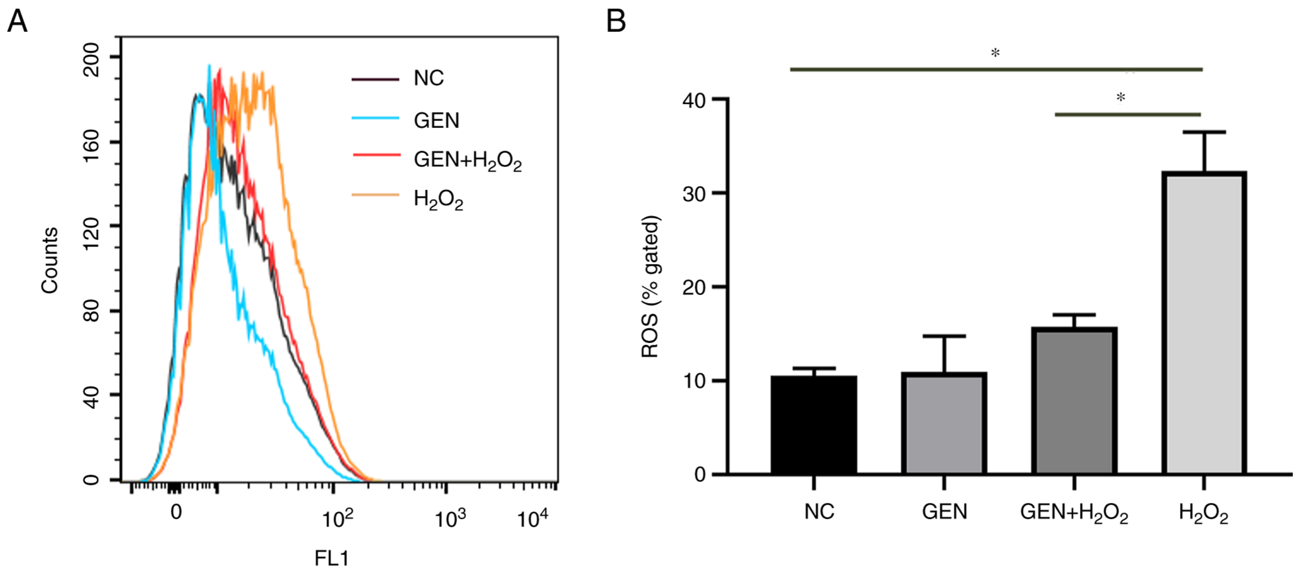

Figure 2. GEN reduces ROS in H_2O_2-damaged cells. (A) Flow cytogram of DCFH-DA single staining. (B) Statistical plot of flow cytometric results. Intracellular reactive oxygen levels increased significantly after H_2O_2 stress. Following GEN pretreatment, the intracellular ROS levels reduced significantly. *P<0.05. GEN, Genistein; ROS, reactive oxygen species.

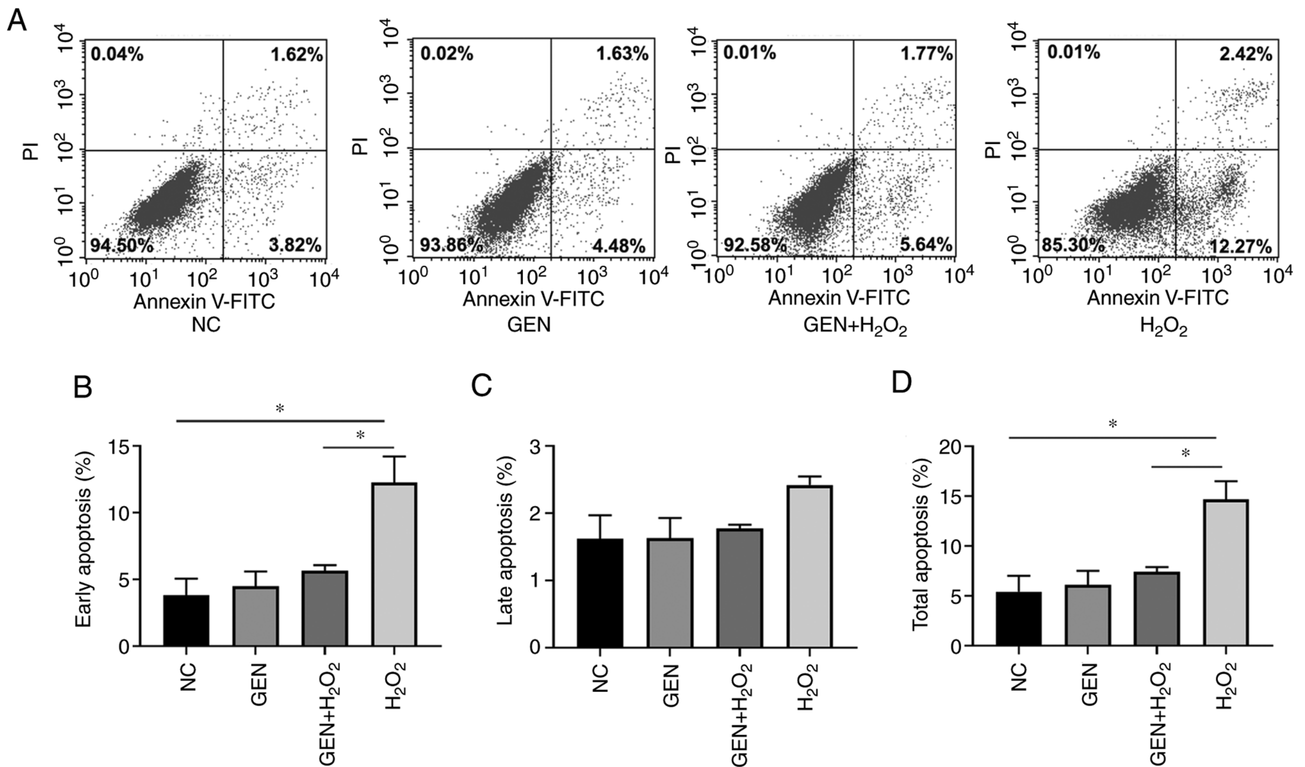

Figure 3. GEN attenuates H_2O_2-induced early apoptosis and total apoptosis. (A) Detection of apoptosis by flow cytometry. (B) Early apoptotic rate in each group. (C) Late apoptotic rate of cells in each group. (D) Total apoptotic rate in each group. GEN reduced the early apoptotic rate and the total apoptotic rate significantly. *P<0.05. GEN, Genistein; NC, negative control.

in Fig. 5A, GEN pretreatment increased the mRNA expression of Nrf2 (**P<0.01), HO-1 (P<0.001) and SOD1 (**P<0.01) simultaneously under H_2O_2 stress. As shown in Fig. 5B and C, compared with the control group, the protein expression of Nrf2 (***P<0.001), HO-1 (***P<0.001) and SOD1 (****P<0.0001) significantly decreased after H_2O_2 stress. Compared with the H_2O_2 group, the protein expression of Nrf2 (*P<0.05), HO-1 (**P<0.01) and SOD1 (*P<0.05) increased significantly after GEN pretreatment.

Effect of GEN on the expression of apoptosis-related genes. To verify the expression of apoptosis-related genes after GEN treatment, the expression of Bax, Bcl-2, and Caspase-3 were detected by RT-qPCR and western blotting, respectively.

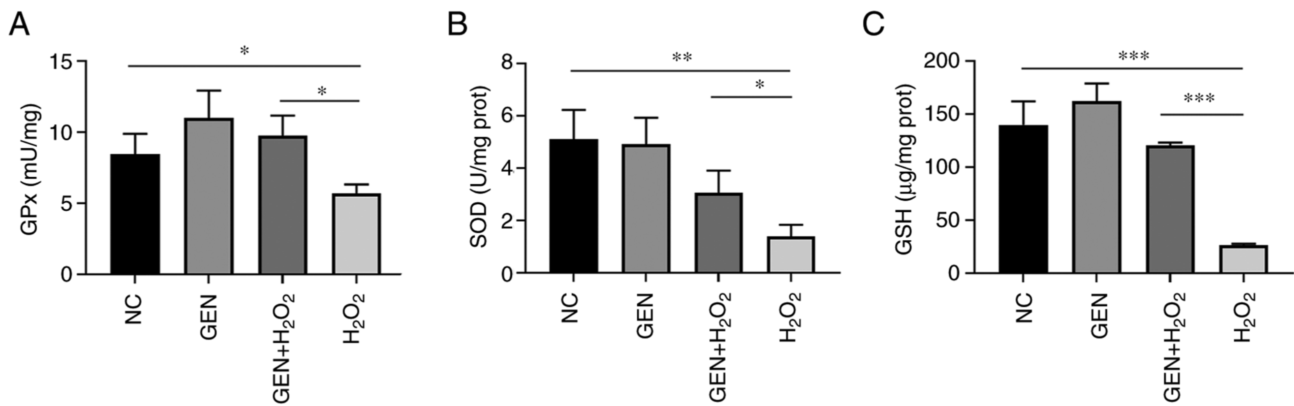

Figure 4. Effect of GEN on the intracellular enzyme activity of HUVECs after H_2O_2 stress. (A) Measurement of GPx activity. (B) Measurement of SOD. (C) Measurement of GSH. The enzyme activity of intracellular GPx was significantly reduced in the H_2O_2 group and increased after GEN pretreatment. The intracellular SOD activity was also significantly reduced and increased after GEN pretreatment. The activity of intracellular GSH was significantly reduced in the H_2O_2 group and increased after GEN pretreatment. *P<0.05, **P<0.01 and ***P<0.001. GEN, Genistein; GPx, glutathione peroxidase; SOD, superoxide dismutase; GSH, reduced glutathione; NC, negative control.

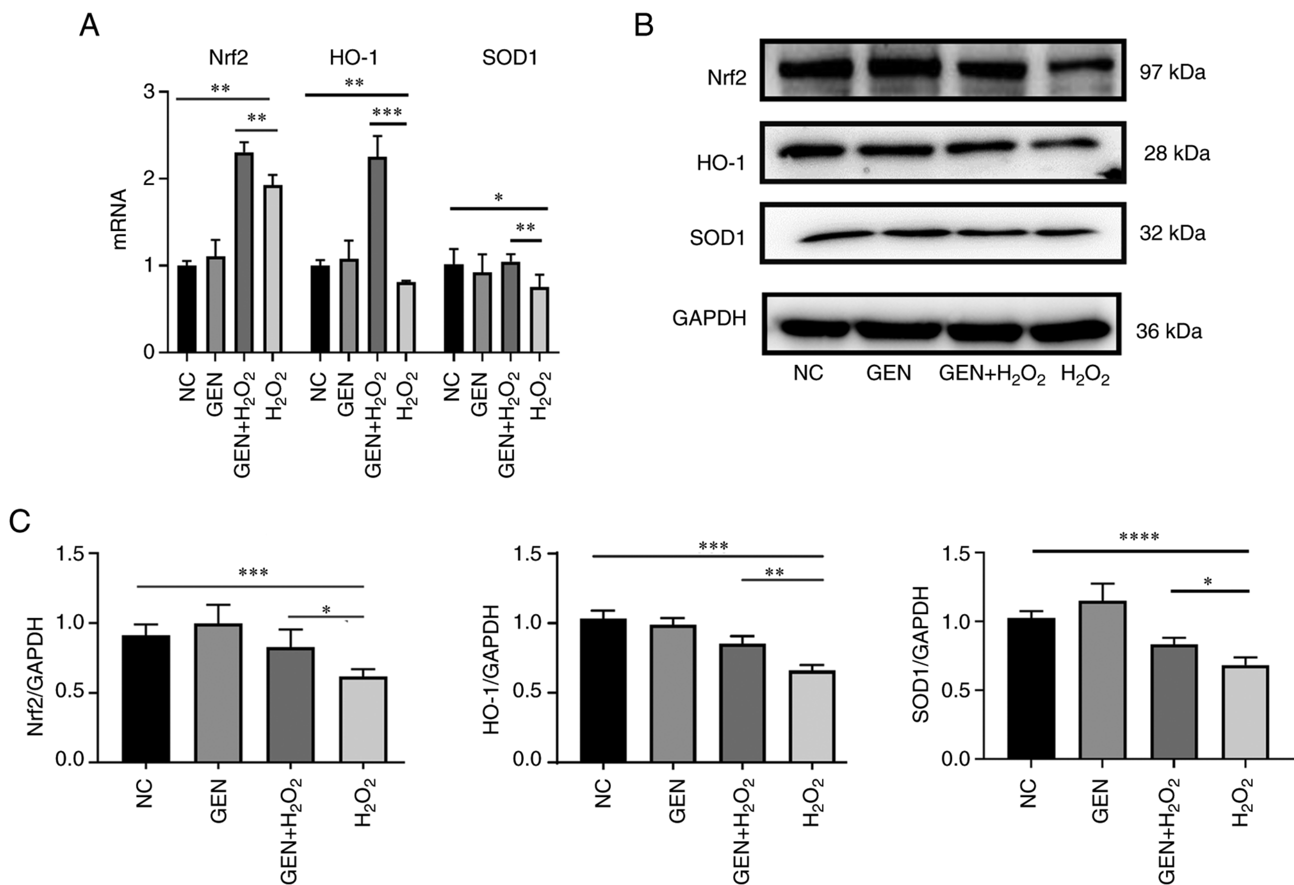

Figure 5. mRNA and protein expression levels of Nrf2, SOD1 and HO-1. (A) The mRNA transcription levels of Nrf2, SOD1 and HO-1 in different groups. (B) Nrf2 pathway-related protein were determined by western blotting. (C) Statistical results of corresponding protein bands. GEN pretreatment increased the mRNA transcription of Nrf2, HO-1 and SOD1 simultaneously under H_2O_2 stress. Compared with the control group, the protein expression of Nrf2, HO-1 and SOD1 decreased after H_2O_2 stress. Compared with the H_2O_2 group, the protein expression of Nrf2, HO-1 and SOD1 increased significantly after GEN pretreatment. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Nrf2, Nuclear factor erythroid 2-related factor 2; SOD1, superoxide dismutase 1; HO-1, heme oxygenase-1; GEN, Genistein; NC, negative control.

As shown in Fig. 6A, compared with the control group, the ratio of Bax/Bcl-2 was significantly higher after H_2O_2 stress (*P<0.05), which was decreased after GEN pretreatment (**P<0.01). The expression of Caspase-3 was also significantly higher in the H_2O_2 group (***P<0.001), and lower in the GEN pretreatment group (***P<0.001; Fig. 6B and C. Compared with the control group, the ratio of Bax/Bcl-2 (***P<0.001) and the protein expression of Caspase-3 were significantly higher (*P<0.05) in the H_2O_2 group. However, the ratio of Bax/Bcl-2 was decreased (P<0.01) and the

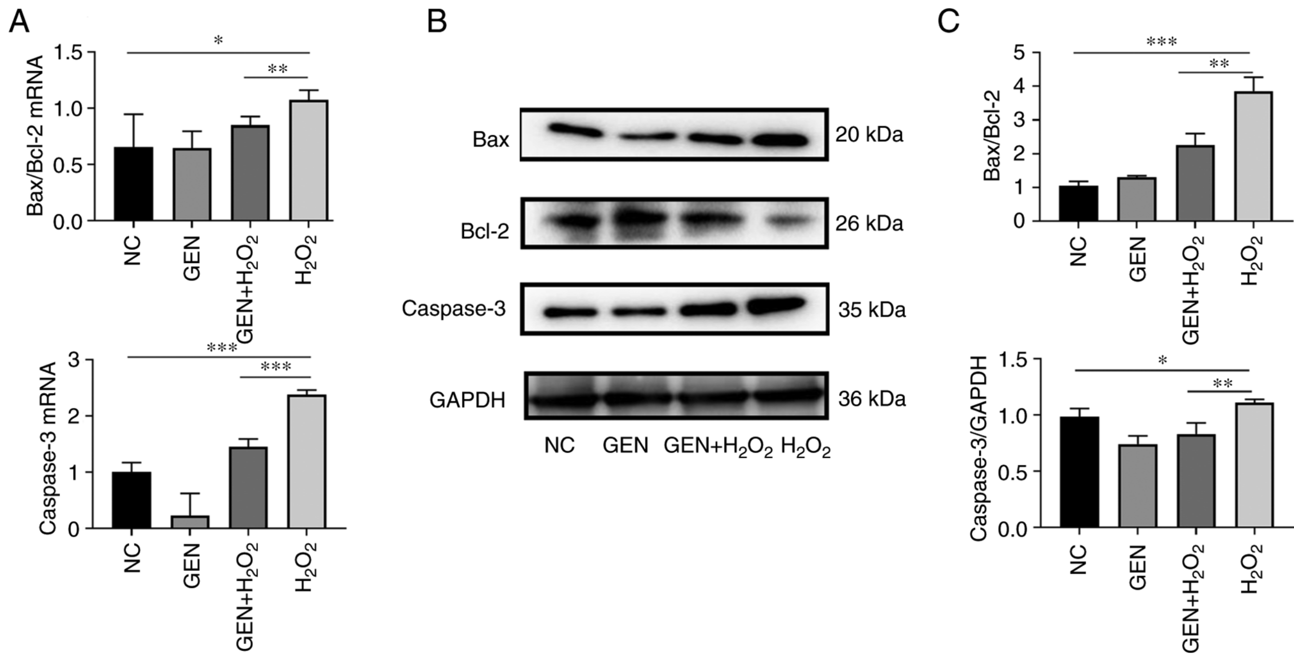

Figure 6. mRNA transcription and protein expression levels of apoptosis-related genes and proteins. (A) The mRNA transcription level of apoptosis-related mRNA. (B) The protein expression level of apoptosis-related proteins. (C) Statistical results of corresponding protein bands. Compared with the control group, the ratio of Bax/Bcl-2 was significantly higher after H_2O_2 stress, which was decreased after GEN pretreatment. The expression of Caspase-3 was also significantly higher in the H_2O_2 group, and lower in the GEN pretreatment group. The results of western blotting revealed that compared with the control group, the ratio of Bax/Bcl-2 and the protein expression of Caspase-3 were significantly higher in the H_2O_2 group. The ratio of Bax/Bcl-2 was decreased and the expression of Caspase-3 was also decreased after GEN pretreatment. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. GEN, Genistein; NC, negative control.

expression of Caspase-3 was also decreased ($^{**}P<0.01$) after GEN pretreatment.

Discussion

AS is a slowly progressive disease which is the pathological basis of coronary heart disease (9,18). Although the progress of clinical treatment has reduced the risk of cardiovascular events, AS and the complications from AS remain the leading cause of death worldwide (10). Of note, the injury of endothelial cells is the most important inducement of AS which is always exacerbated by endothelial dysfunction caused by oxidative stress, inflammation and other factors. Thus, inhibiting the oxidative damage of vascular endothelial cells is an effective way to prevent the occurrence and development of AS. It has been verified that GEN can exabit an antioxidant effects in cancer, post-menopausal syndrome, osteoporosis and a series of cardiovascular diseases (8,11). In the present study, the results further demonstrated that GEN reduced the oxidative damage of HUVECs significantly through the Nrf2/HO-1 signaling pathway.

Cell viability can directly reflect the survival status of cultured cells. In the present study, the results demonstrated that the cell viability was significantly increased after pretreatment with GEN. This result indicated that GEN inhibit the injury induced by oxidation stress partly though the increase of cell viability.

ROS play an important role in pathogen resistance and cell signaling. However, ROS can also have deleterious effects after excessive accumulation (19). Previous studies have shown that the development of AS is closely related to oxidative stress, and excessive ROS can accelerate the process of AS (20,21). H_2O_2 is a common oxidizing agent that always leads to an increase of intracellular ROS and a decrease in the viability of numerous different cells (12). The present study further explored the possible antioxidant effect of GEN. The results of the present study revealed that GEN pretreatment could attenuate the intracellular ROS aggregation under H_2O_2 treatment that is consistent with previous literature (10).

High concentrations of ROS not only cause damage to macromolecules, including DNA (22), but also induces the opening of mitochondrial membrane permeability transition pore to release more ROS (23), which subsequently induce the release of cytochrome C further to cause apoptosis (24). These apoptotic cells will promote the development and progression of coronary artery disease (25). To determine the protective role of GEN after oxidative injury, Annexin-V/FITC double staining was used to further detect apoptosis. It was found that GEN pretreatment could reduce the early and total apoptotic rate of HUVECs after H_2O_2 stress. Meanwhile, the Bcl-2/Bax ratio was increased and the expression of Caspase-3 was correspondingly decreased in the GEN pretreatment group. It has been identified that Caspase-3 is the critical caspase that is also the main effector of the apoptotic program (26). Moreover, apoptosis can be resisted though increased expression of anti-apoptotic proteins (such as Bcl-2) or downregulated expression of pro-apoptotic proteins (such as Bax) (7). Thus, the results of the present study further confirmed that GEN could reduce the apoptosis of HUVECs through decreased expression of Caspase-3 and increased ratio of Bcl-2/Bax after H_2O_2 stress.

Moreover, the activity of SOD, GSH and GPx enzymes was further detected to reveal the effect of GEN on the anti-oxidative ability of vascular endothelial cells. SOD is an enzyme that catalyzes the removal of superoxide radicals (-O_2-) and protects the organism from oxidative damage during physiological aging (27). GSH also plays a key role in protecting cells from oxidative damage and toxicity from exogenous electrophiles to maintain redox homeostasis (28). GPx is another major member of the antioxidant enzyme family, which catalyzes the reduction of peroxides by GSH, scavenges free radicals in the body and thereby reducing the level of ROS. As hypothesized, the activity of all these three enzymes was increased to some extent after GEN pretreatment. These results indicated that H_2O_2 induces the aggregation of intracellular ROS accompanied by decreased activity of intracellular antioxidant system enzymes. However, pretreatment of GEN increased the content of intracellular antioxidant enzymes, which effectively reduced the oxidative damage caused by ROS in cells.

Notably, Nrf2 is a critical cytoprotective factor involved in regulating the expression of antioxidant, anti-inflammatory and detoxification protein genes (29). Nrf2 also plays an integral role as a transcription factor in the maintenance of cellular redox homeostasis and phase II detoxification responses (30,31). Keap1-Nrf2 pathway regulates the expression of a number of cytoprotective genes, including the downstream gene HO-1 (32). HO-1 not only acts as an antioxidant stressor but also regulates a series processes including inflammation, apoptosis, cell proliferation, fibrosis and angiogenesis (29). The present study also revealed that the expression of Nrf2, HO-1 and SOD1 was reduced after H_2O_2 stress. However, GEN pretreatment could reverse the decreased expression of Nrf2, HO-1 and SOD1 significantly. These observations indicated that GEN inhibit the antioxidation injury through the Nrf2/HO-1/SOD1 related pathway.

In summary, the data provided in the present study function as an insight that GEN can exhibit an effective anti-oxidation role though ameliorating the decrease in cell viability and antioxidant ability of vascular endothelial cells after H_2O_2 stress. Moreover, the involvement of Nrf2/HO-1/SOD1 related pathway was further verified to be in H_2O_2-stessed vascular endothelial cells after GEN pretreatment which may be clinically relevant. Understanding the contribution of this molecular network within vascular endothelial cells may provide new therapeutic approaches for the treatment of oxidation damage in AS. The limitations of the present study were the usage of singular cellular model and the lack of *in vivo* experiments, which will be followed up by the authors with an experimental study of an animal model.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81600026,82070034 and 82173572), the Hunan Natural Science Foundation (grant no. 2021JJ30898) and the Research Project of Hunan Provincial Health Commission (grant no B202303027804).

Availability of data and materials

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

Authors' contributions

KX and QQ carried out the experiments, analyzed and interpreted the data, and drafted the manuscript. YY, LY, XD and KZ performed the experiments and statistical analysis. QQ, XW and WW analyzed and interpreted the data, provided the project funding and revised the manuscript. CL analyzed and interpreted the data, revised the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript. KX and CL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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