

Modulation of miRNAs by vitamin C in H₂O₂-exposed human umbilical vein endothelial cells

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Abstract. Vitamin C plays a protective role in oxidative damage by blocking the effects of free radicals. The present study investigated the mechanisms through which vitamin C partly mediates anti-apoptotic and antioxidant functions via the regulation of microRNAs (miRNAs or miRs). For this purpose, a global miRNA expression analysis on human umbilical vein endothelial cells (HUVECs) treated with vitamin C was conducted using microarrays containing human precursor and mature miRNA probes. The results revealed that there were 42 identical miRNAs among the differentially expressed miRNAs in the HUVEC group and H₂O₂ + vitamin C-treated HUVEC group compared to the H₂O₂-exposed HUVEC group, including 41 upregulated miRNAs and 1 down-regulated miRNA. Using bioinformatics analysis, differentially expressed miRNAs were investigated to identify novel target mRNAs and signaling pathways. Pathway enrichment analyses revealed that apoptosis, the mitogen-activated protein kinase (MAPK) signaling pathway, phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and oxidative phosphorylation were significantly enriched. The results from western blot analysis demonstrated that the interleukin (IL)10, matrix metalloproteinase (MMP)2, cAMP-response element binding protein (CREB) and p-CREB protein expression levels in HUVECs transfected with hsa-miR-3928-5p and induced by H₂O₂ were significantly downregulated; the MAPK9, caspase-3 (CASP3) and p-CASP3 protein expression levels in HUVECs transfected with hsa-miR-323a-5p and induced by H₂O₂ were significantly

downregulated. The present study therefore demonstrates that vitamin C partly exerts protective effects on HUVECs through the regulation of miRNA/mRNA axis expression.

Introduction

Aging is considered the main risk factor for various diseases (1); as the aging population increases, the prevalence of several age-related chronic diseases also increases. This is mainly caused by cumulative damage in the organs and cells, thereby leading to the on-set of age-related chronic diseases. Therefore, by exploring the specific mechanisms of aging, valuable information on age-related diseases can be obtained and consequently, strategies to delay its early onset may also be developed.

Oxidative stress is a state in which there are high levels of reactive oxygen species (ROS) compared to antioxidant defenses (2), and this is a hallmark of age-related diseases, including Parkinson's disease (3), Alzheimer's disease (4), chronic inflammation (5), heart failure (6), atherosclerosis (7), kidney disease (8), certain types of cancer (9,10) and aging itself (11). Among these, vascular disease is the most common cause of mortality in the industrialized world (12,13). The normal function and integrity of vascular endothelial cells are of utmost importance for the stability of the vascular environment. The dysfunction of the vascular endothelium, characterized by morphological alterations in the vascular wall, endothelial cell inflammation and apoptosis, is considered the initial event of some diseases (14). Oxidative stress is a critical cause of vascular dysfunction, as ROS can damage cells of the vascular wall and induce vascular endothelial cell dysfunction (15). Antioxidants prevent the damage of vascular endothelial cells and are considered one of the crucial factors for the prevention of cardiovascular diseases (16).

Antioxidants are highly effective in the prevention or treatment of oxidative damage in animal models (17). Commonly used antioxidants include vitamin C, α -tocopherol and polyphenols (18). Among these, vitamin C, a recognized antioxidant, is easily accessible in daily life and has the advantages of low cost, a significant effect, easy accessibility and hydrophilicity (19). As a potent antioxidant, vitamin C plays a protective role in oxidative damage

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by blocking the production of free radicals (20,21). It also plays a critical role in certain types of cancer and exerts immunomodulatory effects that enhance host defenses (22). Vitamin C deficiency can lead to scurvy, a fatal disease (23). Vitamin C suppresses the expression of KRAS and BRAF, which inhibit glycolysis and the ensuing energy crisis by targeting GAPDH in colorectal cancer cells (24). In addition, high levels of vitamin C have been shown to induce the apoptosis of HT29 colon cancer and MCF7 breast cancer cells by hindering the energy flux in the tricarboxylic acid cycle and glycolysis, eventually resulting in the insufficient production of adenosine triphosphate (25). Vitamin C can inhibit the death of human umbilical vein endothelial cells (HUVECs) induced by oxidative stress (19). Vitamin C or vitamin C-Na pre-treatment has also been shown to enhance antioxidant capacity, thus protecting H9C2 cells from heat-induced damage (26). It also improves the endothelium-dependent vasodilation of forearm resistance vessels in patients with hypercholesterolemia (27). Vitamin C reduces lipid peroxidation and enhances the antioxidant defense system, which exerts a beneficial effect on the heart by reducing oxidative stress in patients with cardiovascular disease (28). However, the molecular mechanisms of vitamin C in vascular-related diseases warrant further investigation.

MicroRNAs (miRNAs or miRs) are a family of small non-coding RNA molecules with 18-25 nucleotides that bind to the 3'-untranslated regions of target mRNAs in a sequence-specific manner, inhibiting their translation or regulating their degradation (29). The expression of miRNAs is tissue-specific, and this localized expression is crucial for their tailored roles in regionalized function and development (30). miRNAs play vital roles in the absorption of vitamin C in the intestines by modulating the post-transcriptional regulation of several vitamin transporter genes, including that of solute carrier family (*SLC*)23A2, *SLC19A2*, *SLC52A3*, *SLC26A3* and *SLC15A1* (31). Vitamin C can also regulate multiple stem cell-specific signaling pathways, such as cell adhesion molecules, fatty acid biosynthesis and hormone signaling pathways by altering miRNA expression (32).

Although vitamin C has been widely studied, the mechanisms underlying the protective effects of vitamin C on H₂O₂-induced HUVECs associated with vascular diseases warrant further investigation. The present study demonstrates the potential benefits of vitamin C treatment against H₂O₂-induced oxidative damage in HUVECs, indicating that vitamin C partly exerts protective effects against H₂O₂-induced oxidative damage by regulating the expression of miRNAs.

Materials and methods

Culture and treatment of HUVECs. HUVECs were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in complete endothelial cell growth medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (100 U/ml; BD Biosciences). HUVECs were digested with 0.25% trypsin and 1x10⁴ cells/ml HUVECs were seeded and cultured at 37°C under 5% CO₂. In the H₂O₂ + vitamin C-treated HUVEC group, the HUVECs were cultured in endothelial cell growth medium containing

100 μM H₂O₂ (Sigma-Aldrich; Merck KGaA) for 2 h and were then cultured in endothelial cell growth medium supplemented with 200 μM vitamin C (Sigma-Aldrich; Merck KGaA) for 48 h. In the H₂O₂-treated HUVEC group, the HUVECs were cultured in endothelial cell growth medium containing 100 μM H₂O₂ for 2 h and were then cultured in endothelial cell growth medium for 48 h. HUVECs not treated with vitamin C and H₂O₂ were used as control.

Apoptosis assays. The HUVECs from each group were washed twice with cold phosphate-buffered saline before being resuspended in 1X Binding Buffer at a concentration of 1x10⁶ cells/ml. The cells were stained with FITC-conjugated anti-Annexin V antibody and propidium iodide (PI) in the apoptosis kit (cat. no. 556547, BD Biosciences) for 15 min at room temperature and dark, and apoptotic cells were quantified using a FACSCalibur flow cytometer (BD Biosciences).

Measurement of ROS generation. According to the manufacturer's protocol, the ROS assay kit (S0033; Beyotime Institute of Biotechnology, Inc.) was used to measure intracellular ROS levels using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, cells treated with 100 μM H₂O₂ for 2 h or 100 μM H₂O₂ for 2 h + 200 μM vitamin C for 48 h were washed twice with PBS. The cells were then incubated with 10 μM DCFH-DA at 37°C for 30 min. After washing with PBS twice, the fluorescence of the cells was imaged using a confocal microscope (Carl Zeiss AG) with an excitation of 488 nm/emission of 529 nm. The fluorescence intensity was measured using ImageJ_v1.8.0 software.

Analysis of the expression of miRNAs. miRNAs were isolated using a miRNA isolation kit (Guangzhou Forevergen Biosciences Co., Ltd.) that specifically captures small RNAs with lengths of <200 nucleotides. The quality of the RNA samples was examined on an Agilent 2100 BioAnalyzer (Agilent Technologies, Inc.) and the yield of the RNA samples was determined using the ABI Step One Plus Real-Time PCR System (Applied Biosystems). The miRNA profile was analyzed for hierarchical clustering to produce heatmaps.

Gene ontology (GO) and pathway enrichment analyses of differentially expressed miRNAs. GO and pathway enrichment analyses were performed to identify biological processes that were potentially regulated by the differentially expressed miRNAs based on the GO (<http://geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<http://www.genome.jp/kegg/pathway.html>) databases. The P-value for each GO term was calculated using the right-sided hypergeometric tests. The Benjamin-Hochberg adjustment was used for multiple test correction (33,34). Those terms with a P-value <0.05 were considered to be significantly enriched. Simultaneously, the target mRNAs associated with apoptosis or oxidative metabolic signaling pathways of the top 10 differentially expressed miRNAs were predicted using TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>). The top 10 differentially expressed miRNAs and their target mRNAs associated with cell apoptosis or involved in oxidative metabolism were integrated, and regulatory networks were constructed using Cytoscape 3 software.

Reverse transcription-quantitative PCR (RT-qPCR) validation analyses of miRNAs. Total RNA was extracted from the cells and gene expression was detected by RT-qPCR. According to the manufacturer's protocol, cDNA was synthesized from total RNA using M-MLV Reverse Transcriptase (Promega Corporation). The GoTaq qPCR Master Mix (Promega Corporation) was used for qPCR. The ABI 7500 system (Applied Biosystems) was used to perform the PCR amplification, while U6 was used as an internal control. The primer information of the miRNAs is presented in Table SI.

Transient transfection. Negative control (NC) mimics, hsa-miR-323a-5p mimics and hsa-miR-3928-5p mimics were purchased from GenePharma. The sequences of the mimics were as follows: hsa-miR-323a-3p mimics sense, 5'-CAC AUUACACGGUCGACCUCU-3' and antisense, 5'-AGG UCGACCGUGUAAUGUGUU-3'; hsa-miR-3928-5p mimics sense, 5'-UGAAGCUCUAAGGUUCCGCCUGC-3' and antisense, 5'-AGGCGGAACCUUAGAGCUUCAUU-3'; NC mimics sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. HUVECs were transfected with 50 nM hsa-miR-323a-3p mimics, 50 nM hsa-miR-3928-5p mimics, or 50 nM NC mimics using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Following transfection for 48 h, the cells were incubated with 100 μ M H₂O₂ for 2 h and harvested for western blot analysis.

Western blot analysis. Cells were lysed using the RIPA buffer (R0010; Solarbio) to separate the protein. The BCA working solution was used to detect the protein concentration at 562 nm using BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 10% SDS-PAGE was used to isolate the target protein. The isolated target protein was transferred to a PVDF membrane. After sealing with 5% non-fat milk at room temperature for 1 h, primary antibodies, including anti-interleukin (IL)10 (1:1,000; ab34843, Abcam), anti-cAMP-response element binding protein (CREB; 1:1,000; ab31387, Abcam), anti-p-CREB (1:1,000; CST9198, Cell Signaling Technology, Inc.), anti-caspase (CASP)3 (1:1,000; CST9662, Cell Signaling Technology, Inc.), anti-cleaved-CASP3 (1:1,000; CST9662, Cell Signaling Technology, Inc.), anti-mitogen-activated protein kinase (MAPK)9 (1:1,000; CST4672, Cell Signaling Technology, Inc.), anti-matrix metalloproteinase (MMP)2 (1:1,000; ab37150, Abcam) and anti-GAPDH (1:1,000; 60004-1-Ig, Proteintech) were incubated with the PVDF membrane overnight at a temperature 4°C. HRP-labeled secondary antibody (1:5,000; ab150117, Abcam) was then used to incubate the membrane for 2 h at room temperature. GAPDH was used as an internal control. The target protein levels were visualized by an enhanced chemiluminescence reagent (Vazyme Biotech Co., Ltd.). ImageJ v1.8.0 software (National Institutes of Health) was used for the semi-quantitative analysis of protein expression.

Statistical analyses. The results are shown as the means \pm standard deviation from at least 3 independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test evaluations for multiple comparisons with SPSS 19.0 statistical

software. A P<0.05 was considered to indicate a statistically significant difference.

Results

Vitamin C significantly reduces the H₂O₂-induced apoptosis of HUVECs. As a potent first-line antioxidant, vitamin C attenuates the production of free radicals and reduces oxidative damage (20,21). In the present study, to further investigate the antioxidant function of vitamin C, vitamin C was used to treat H₂O₂-induced HUVECs. The percentage of apoptotic HUVECs induced with H₂O₂ was approximately 6%, while vitamin C significantly reduced the percentage of apoptotic HUVECs induced by H₂O₂ (Fig. 1A and B). In addition, ROS assay revealed that vitamin C reduced the green fluorescence intensity in HUVECs induced with H₂O₂ (Fig. 1C and D). These results demonstrated that vitamin C attenuated H₂O₂-induced apoptosis and oxidative damage in H₂O₂-induced HUVECs.

Vitamin C modulates miRNA profiles in H₂O₂-induced HUVECs. To identify differentially expressed miRNAs due to vitamin C treatment, a comprehensive miRNA microarray analysis of samples from H₂O₂-induced HUVECs with or without vitamin C treatment was conducted. These miRNAs in HUVECs were isolated after 2 h of H₂O₂ exposure and 48 h of vitamin C treatment. The miRNAs exhibited a significant (P<0.05) 1.5-fold difference in expression following treatment with H₂O₂ + vitamin C compared with the control group and H₂O₂ exposure group, respectively. The results revealed that there was a significant change in the expression of 287 miRNAs, including 70 upregulated miRNAs and 217 miRNAs that were downregulated in the H₂O₂ exposure group compared with the control group (Fig. 2A and Table SII). In addition, in the H₂O₂ + vitamin C treatment group, 710 (including 706 upregulated and only 4 downregulated) miRNAs were differentially expressed compared with the H₂O₂ treatment group (Fig. 2B and Table SIII). The analyses revealed that there were 42 identical miRNAs among the differentially expressed miRNAs induced in the HUVEC group and H₂O₂ + vitamin C-treated group compared to the H₂O₂-treated group, including 41 upregulated miRNAs and 1 downregulated miRNAs (Fig. 2C and Table SIV). Hierarchical cluster analysis using the normalized miRNA expression data confirmed that the expression of miRNAs in the H₂O₂ or H₂O₂ + vitamin C-treated HUVECs could be clearly distinguished from that in the control group (Fig. 2).

GO analysis and KEGG pathway prediction. Categorizing these miRNAs may enhance our understanding of the cellular components and biological processes regulated by the miRNAs with an altered expression in the treated HUVECs. GO enrichment analyses demonstrated that these differentially expressed miRNAs were associated with cellular components and biological processes, including cell, organelle, membrane, protein-containing complex, membrane-enclosed lumen, catalytic activity, binding, localization, metabolic processes, developmental processes, multicellular organismal processes, immune system processes, positive and negative regulation biological processes, and cellular proliferation (Fig. 3A).

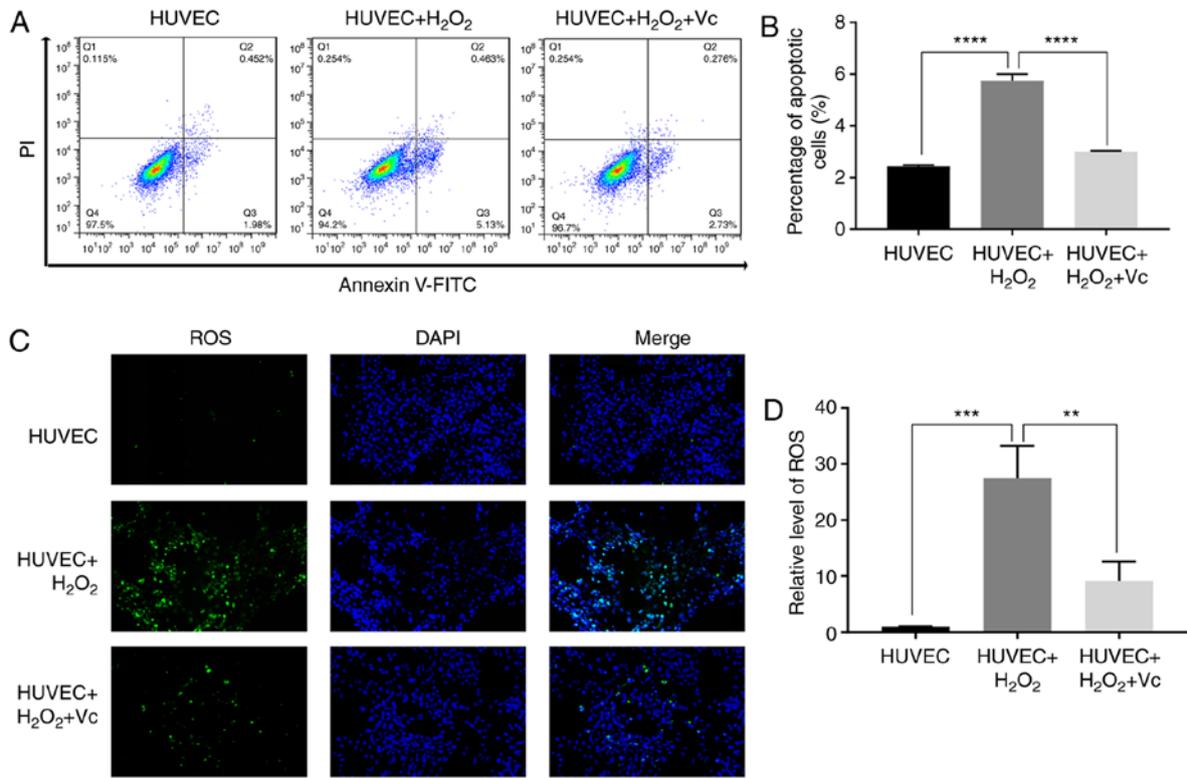


Figure 1. Vitamin C reduces apoptosis and oxidative damage in human umbilical vein endothelial cells (HUVECs) induced by H₂O₂. (A) The apoptosis rate of HUVECs treated with 100 μM H₂O₂ for 2 h or 100 μM H₂O₂ for 2 h + 200 μM vitamin C for 48 h was analyzed by flow cytometry. (B) Analysis of the percentage of apoptotic HUVECs in (A). (C) ROS expression level in HUVECs induced by 100 μM H₂O₂ for 2 h or 100 μM H₂O₂ for 2 h + 200 μM vitamin C for 48 h was observed using a confocal microscope. (D) Semi-quantitative analysis of ROS fluorescence intensity in (C). Data are shown as the means ± standard deviation (n=3). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. **P<0.01, ***P<0.001, ****P<0.0001.

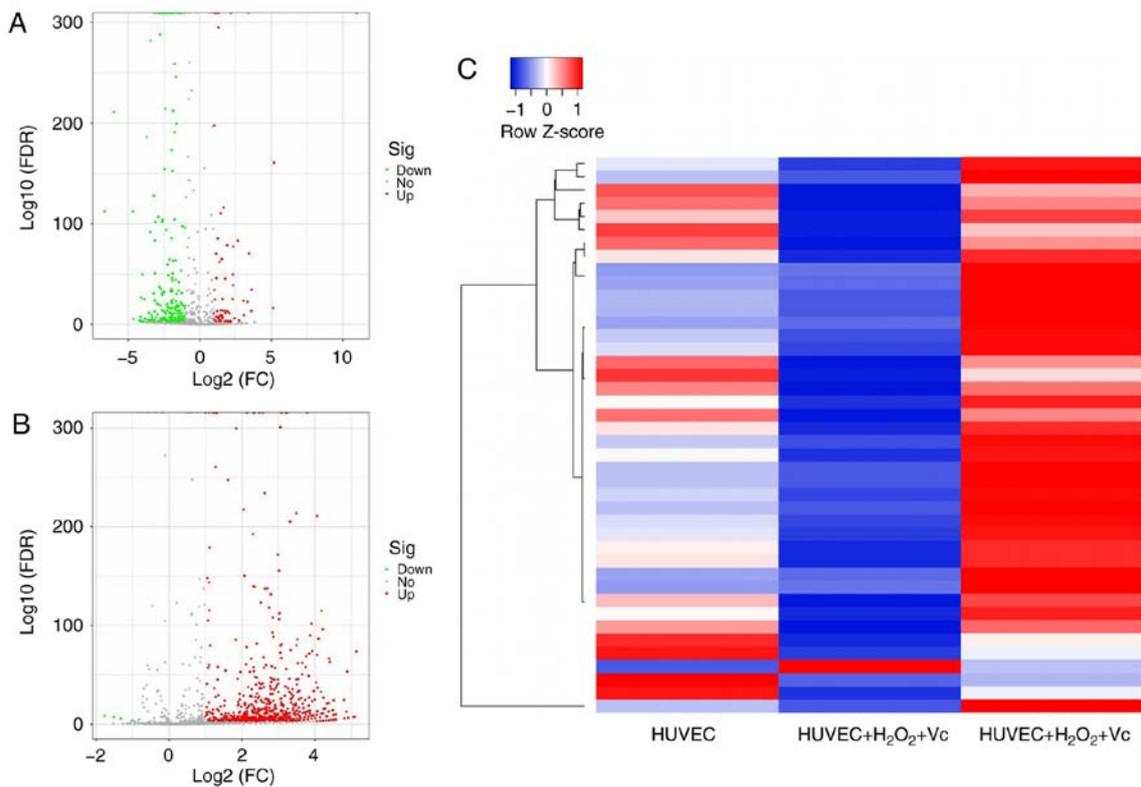


Figure 2. Differential miRNA expression in human umbilical vein endothelial cells (HUVECs) following exposure to H₂O₂ and vitamin C. (A) Significant alterations of miRNA levels in HUVECs following exposure to 100 μM H₂O₂ for 2 h. (B) Significant alterations in miRNA levels in HUVECs following exposure to 100 μM H₂O₂ for 2 h + 200 μM vitamin C for 48 h. (C) Cluster heatmap of the same miRNAs among the differentially expressed miRNAs in HUVECs induced by H₂O₂ or vitamin C + H₂O₂.

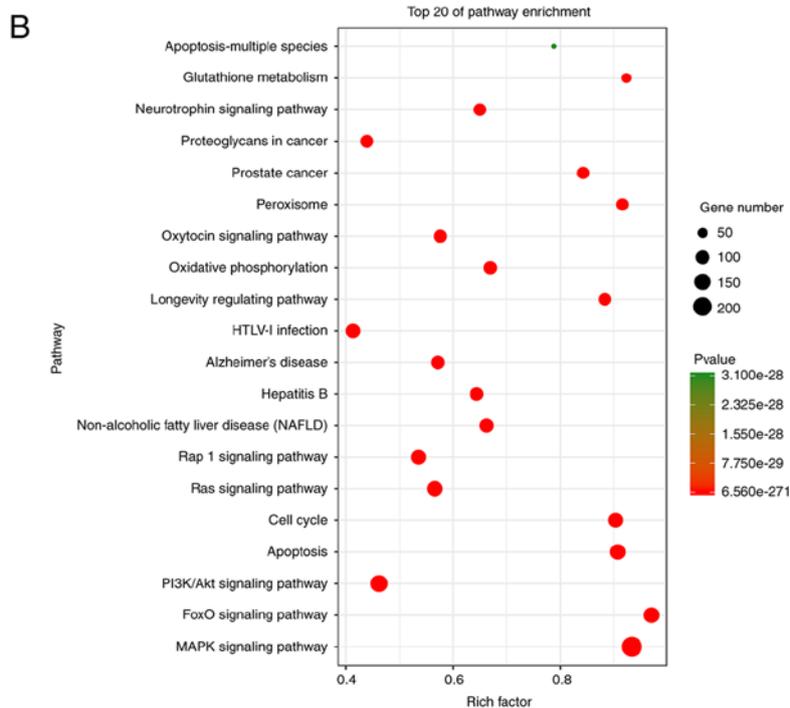
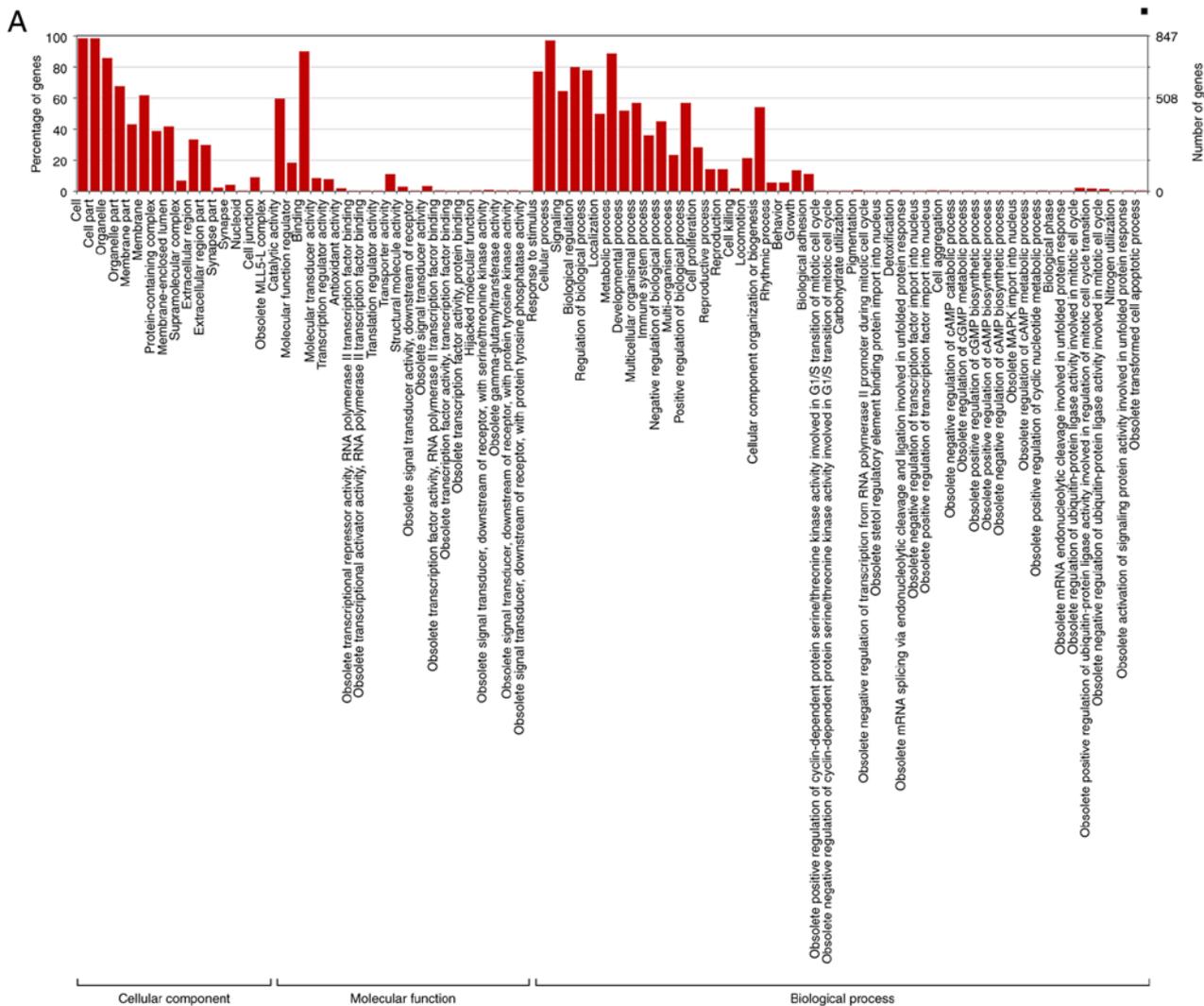


Figure 3. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway prediction. (A) GO biological pathways and (B) KEGG biological pathways were potentially affected by the same differentially expressed miRNAs following both H₂O₂ treatment and vitamin C + H₂O₂ treatment of human umbilical vein endothelial cells (HUVECs).

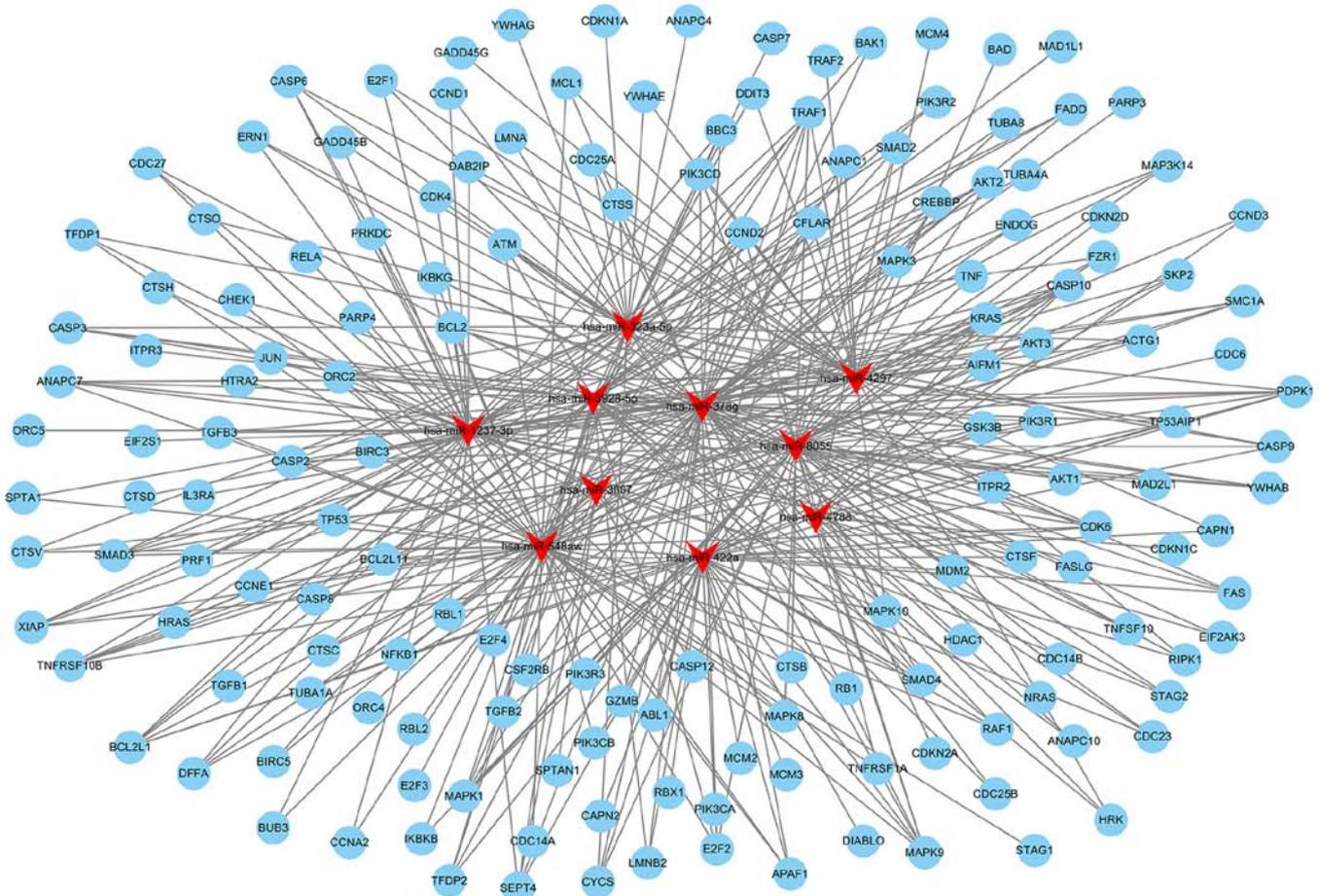


Figure 4. The top 10 differentially expressed miRNAs and their target mRNAs associated with cell apoptosis were integrated, and regulatory networks were constructed using Cytoscape 3 software. The red triangles represent miRNAs and the blue rounds represent the target mRNAs.

Pathway enrichment analyses revealed significant enrichment in apoptosis, MAPK signaling pathway, PI3K/Akt signaling pathway and oxidative phosphorylation (Fig. 3B).

miRNA target prediction by bioinformatics analysis. The top 10 miRNAs with the most evident differences in expression were hsa-miR-323a-5p, hsa-miR-378g, hsa-miR-4788, hsa-miR-4297, hsa-miR-422a, hsa-miR-3928-5p, hsa-miR-3687, hsa-miR-1237-3p, hsa-miR-8055 and hsa-miR-548aw. The target mRNAs associated with apoptosis or oxidative metabolic signaling pathways of the top 10 differentially expressed miRNAs were predicted using TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>). The network of miRNA-targeted mRNAs was constructed according to their regulatory association. hsa-miR-323a-5p can target CASP3, CASP6, CASP9, MAPK9 and other mRNAs to regulate apoptosis; hsa-miR-422a may be associated with apoptosis by regulating PIK3CA, E2F2, FAS, or SMAD4; hsa-miR-8055 may target BCL2, FAS, TNF and other mRNAs to regulate apoptosis (Fig. 4 and Table SV). hsa-miR-3928-5p may be involved in regulating oxidative stress by targeting IL10, MMP2, or CREB; hsa-miR-378g may target ATG12, ANPEP, MAPK3, or EREG to mediate oxidative stress; hsa-miR-1237-3p may target AKT2, AKT3 and MAPK to regulate oxidative stress (Fig. 5 and Table SV).

Validation of differentially expressed miRNAs. To confirm the miRNA microarray data, RT-qPCR was performed to examine the expression levels of the top 10 miRNAs from the H₂O₂ or H₂O₂ + vitamin C-treated HUVEC groups. The expression levels of hsa-miR-8055, hsa-miR-3928-5p, hsa-miR-323a-5p, hsa-miR-378g, hsa-miR-422a and hsa-miR-1237-3p detected by RT-qPCR were downregulated in the HUVECs induced by H₂O₂ compared to those in the control HUVECs, and were upregulated in the HUVECs treated with vitamin C and H₂O₂ compared to those in the HUVECs exposed to H₂O₂; these findings were consistent with those of the microarray analyses (Fig. 6 and Table SIV). However, the hsa-miR-548aw and hsa-miR-4297 expression levels were upregulated in the HUVECs induced by H₂O₂ compared to those in the control HUVECs, and were downregulated in the HUVECs treated with vitamin C and H₂O₂ compared to those in the HUVECs treated with H₂O₂; the hsa-miR-4788 and hsa-miR-3687 expression levels were upregulated in HUVECs induced by H₂O₂ compared to those in HUVECs; these findings were inconsistent with the results of microarray analysis. Overall, the results of RT-qPCR verification of the selected miRNAs were mostly consistent with the results of sequencing analysis.

Role of hsa-miR-3928-5p and hsa-miR-323a-5p in oxidation and apoptosis of HUVECs. To further analyze the molecular

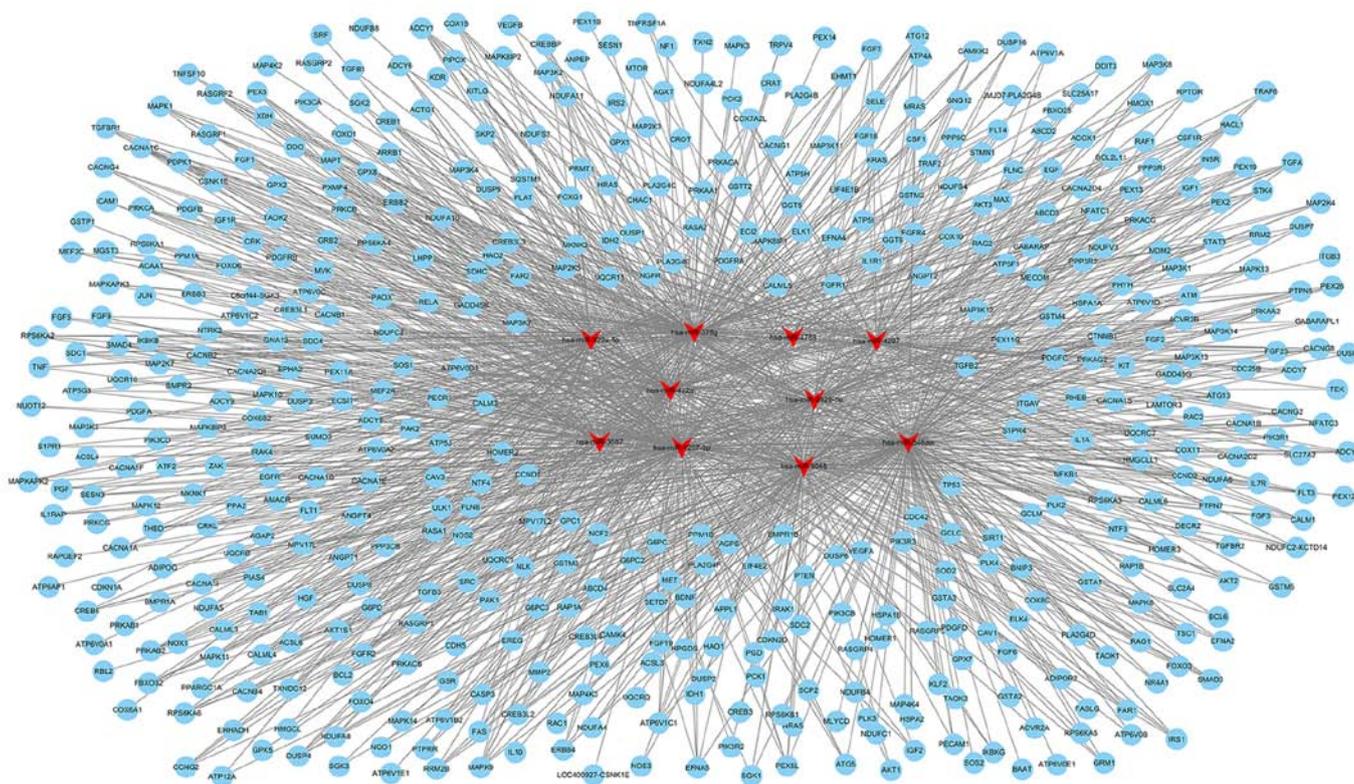


Figure 5. The top 10 differentially expressed miRNAs and their target mRNAs involved in oxidative metabolism were integrated, and regulatory networks were constructed using the Cytoscape 3 software. The red triangles represent miRNAs and the blue rounds represent the target mRNAs.

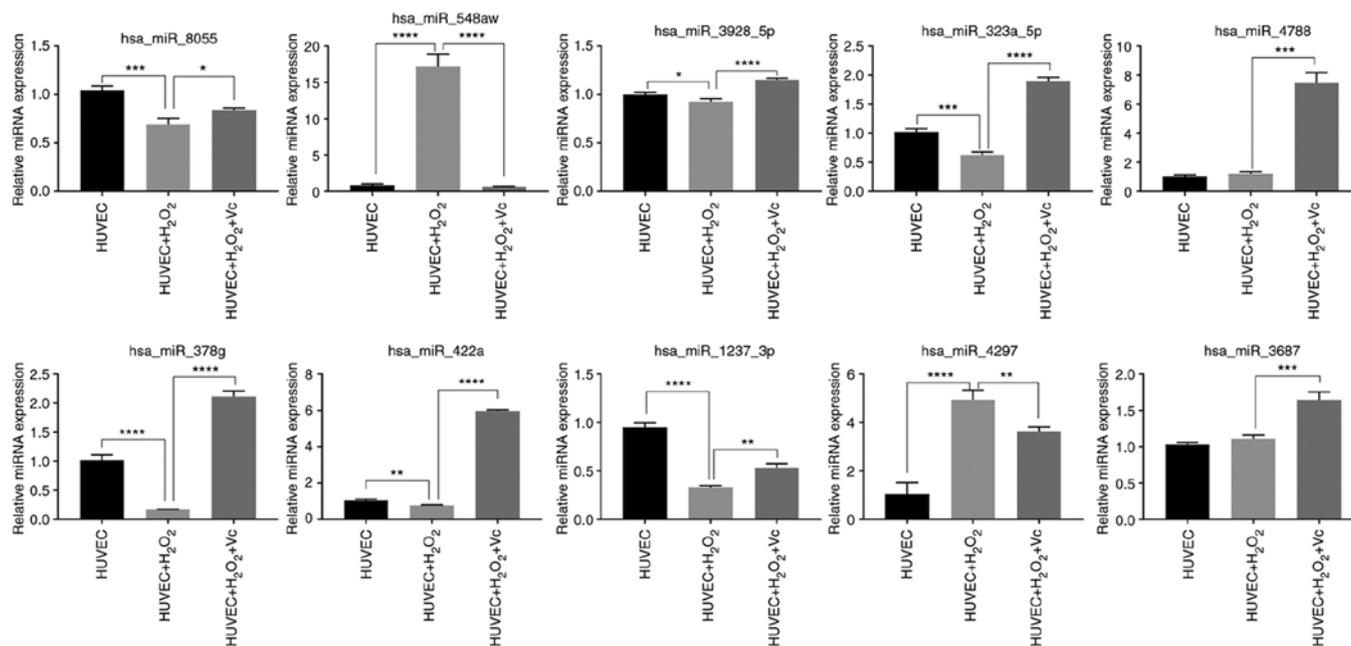


Figure 6. RT-qPCR analysis of the changes in miRNA expression in H_2O_2 -induced and vitamin C + H_2O_2 -treated human umbilical vein endothelial cells (HUVECs). Data are shown as the means \pm standard deviation (n=3). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

mechanisms of miRNAs in apoptosis and oxidation, western blot analysis was used to analyze the expression level of mRNAs regulated by hsa-miR-3928-5p and hsa-miR-323a-5p. The results revealed that H_2O_2 upregulate the IL10, MMP2, CREB and p-CREB protein expression levels, and the ratio

of p-CREB/CREB in HUVECs; however, the IL10, MMP2, CREB and p-CREB protein expression levels and the ratio of p-CREB/CREB in the HUVECs transfected with has-miR-3928-5p and induced by H_2O_2 were significantly downregulated, compared to those of HUVECs induced

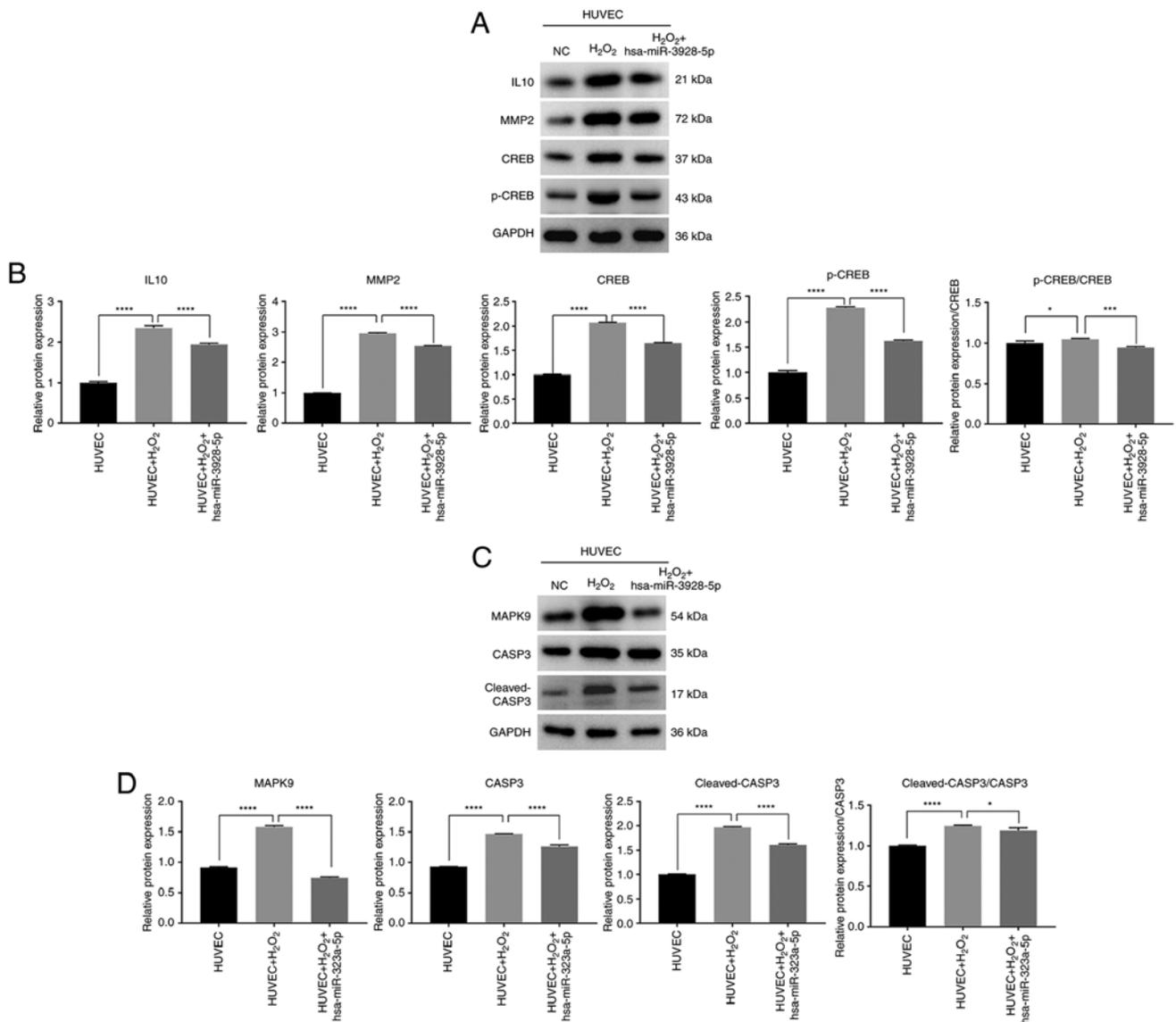


Figure 7. Effect of hsa-miR-3928-5p and hsa-miR-323a-5p on related target mRNAs expression levels in human umbilical vein endothelial cells (HUVECs) induced by H₂O₂ examined by western blot analysis. (A) IL10, MMP2, CREB and p-CREB protein expression levels and the ratio of p-CREB/CREB in HUVECs under different processing conditions. (B) Semi-quantitative analysis of the protein expression levels in (A). (C) MAPK9, CASP3 and cleaved-CASP3 protein expression levels and the ratio of cleaved-CASP3/CASP3 in HUVECs under different processing conditions. (D) Semi-quantitative analysis of the protein expression levels in (C). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-hoc test. **P*<0.05, ****P*<0.001, *****P*<0.0001. IL, interleukin; MMP2, matrix metalloproteinase; CREB, cAMP-response element binding protein; CASP3, caspase-3.

by H₂O₂ (Fig. 7A and B). Simultaneously, the results also revealed that H₂O₂ upregulated the MAPK9, CASP3 and cleaved-CASP3 protein expression levels and the ratio of cleaved-CASP3/CASP3 in HUVECs (Fig. 7C and D). Moreover, the MAPK9, CASP3 and cleaved-CASP3 protein expression levels and the ratio of cleaved-CASP3/CASP3 in HUVECs transfected with hsa-miR-323a-5p and induced by H₂O₂ were significantly downregulated, compared to those of HUVECs induced by H₂O₂ (Fig. 7C and D). It could thus be inferred that vitamin C may regulate the oxidation and apoptosis of HUVECs through the above-mentioned molecular mechanism.

Discussion

Vitamin C is known to be a potent antioxidant that quenches ROS and has also been demonstrated to ease vascular endothelium

dysfunction in conditions, such as hyperhomocysteinemia, diabetes, hypercholesterolemia, coronary artery disease, and renovascular hypertension (27,35-38). Vitamin C induces the pluripotent differentiation of mouse embryonic stem cells via the modulation of miRNA expression (39). High Vitamin C levels result in enhanced anti-atherosclerotic and anti-senescence effects by regulating anti-inflammatory miRNAs (40,41). At present, the role of vitamin C-dependent miRNAs in the regulation of the antioxidant and antiapoptotic activities of endothelial cell remains to be fully determined. Hence, the identification of vitamin C-induced differentially expressed miRNAs is crucial for the further understanding of the specific mechanisms underlying endothelial dysfunction. In the present study, a list of differentially expressed miRNAs were identified following vitamin C treatment and it was revealed that vitamin C attenuated the apoptosis and oxidative damage of H₂O₂-induced HUVECs.

Simultaneously, GO analysis demonstrated that cellular components and biological processes were clearly critical to the H₂O₂-induced oxidative stress and apoptosis of HUVECs. Moreover, KEGG annotation demonstrated that apoptosis, the MAPK signaling pathway, PI3K/Akt signaling pathway and oxidative phosphorylation were involved in the anti-oxidative and anti-apoptotic effects of vitamin C in H₂O₂-induced HUVECs. Research has indicated that all MAPK inhibitors increase the O₂^{•-} levels in H₂O₂-induced A549 cells (42). The PI3K/Akt signaling pathway is related to survival, angiogenesis and oxidative stress under pathophysiological conditions in ischemia (43). Klotho has been shown to weaken oxidized low-density lipoprotein (ox-LDL)-induced oxidative stress in HUVECs via the upregulation of oxidative scavengers by suppressing lectin-like ox-LDL receptor expression and activating the PI3K/Akt/eNOS pathway (44). To further investigate the potential roles of miRNAs involved in HUVECs following exposure to vitamin C, the present study analyzed the predicted target mRNAs of selected miRNAs. The present study focused on the top 10 miRNAs, including hsa-miR-323a-5p, hsa-miR-378g, hsa-miR-4788, hsa-miR-4297, hsa-miR-422a, hsa-miR-3928-5p, hsa-miR-3687, hsa-miR-1237-3p, hsa-miR-8055 and hsa-miR-548aw, with the most evident differences in expression. It was revealed that these miRNAs target mRNAs that regulate cell apoptosis and oxidative metabolism signaling pathways.

miR-422a can inhibit the migration and proliferation of gastric cancer cells, and can promote the metabolic transition from aerobic glycolysis to oxidative phosphorylation (45). miR-323a-3p can attenuate the apoptosis of 16HBE14o-cells stimulated with staurosporine or tunicamycin by inhibiting the CASP3 expression level (46). CASP-9/3 plays a critical role in apoptotic signaling in the mitochondria (47). The activation of CASP-9/3 mediates cell apoptosis in various cell types (48-50). In addition, 1,25-dihydroxyvitamin-D₃ induces neutrophil apoptosis in periodontitis with type 2 diabetes mellitus patients via the p38/MAPK pathway (51). In this study, western blot showed that hsa-miR-323a-5p downregulated the protein expression levels of CASP3, Cleaved-CASP3, and MAPK9 and the ratio of the Cleaved-CASP3/CASP3 in HUVECs induced by H₂O₂, suggesting that hsa-miR-323a-5p obtained the antiapoptotic effects of Vitamin C by targeting CASP3 and MAPK9. In oxidative stress, MMP2 knockdown has been shown to prevent the protective effects of miR-125 inhibitor on H9C2 cells (52). MMPs play important roles in anti-inflammation; the activity of MMP-2 is increased by oxidative stress in early hypertension (53). The administration of anthocyanin suppresses the generation of ROS and attenuates naproxen-induced suppression of MMP-2 (54). IL10, a human cytokine influencing immunoregulation and inflammation, has anti-inflammatory properties and plays an important role in limiting immune responses to pathogens and oxidative stress (55). The activation of the Akt/CREB axis by stressin-1 can counteract the adverse effects of various cell stresses (56). In the present study, the results of western blot analysis revealed that hsa-miR-3928-5p downregulated the protein expression levels of IL10, MMP2, CREB and p-CREB, and the ratio of p-CREB/CREB in HUVECs induced by H₂O₂, suggesting that hsa-miR-3928-5p mediated the anti-inflammatory effects of vitamin C by targeting IL10, MMP2 and CREB.

In conclusion, the present study may be an important step in obtaining a greater understanding of the mechanisms through which vitamin C exerts anti-apoptotic and antioxidant effects via miRNA signaling networks, thereby revealing the potential molecular mechanisms of vitamin C as regards the antioxidation and apoptosis of HUVEC induced by H₂O₂.

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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

JW, YHu and YHuang conceived and designed the experiments. JW, JingjingL, MLi, MLin, LM, XH, JianqiuL performed the experiments. JW and JingjingL analyzed the data and wrote the manuscript. JianqiuL and YHuang revised the manuscript. All authors have read and approved the final 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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