**Regular Article**

**Epimedin C Protects H₂O₂-Induced Peroxidation Injury by Enhancing the Function of Endothelial Progenitor HUVEC Populations**

Dan-Hong Wei, a Jiu-Ling Deng, b Rong-Zhen Shi, b Li Ma, b Jia-Man Shen, b Robert Hoffman, b,c Ying-Hong Hu, a Hui Wang,* b and Jian-Li Gao* b

a Department of Neuroscience Care Unit, the Second Affiliated Hospital, Zhejiang University School of Medicine; Hangzhou, Zhejiang 310009, China; b Zhejiang Chinese Medical University; Hangzhou, Zhejiang 310053, China; and c Five Branches University, San Jose, CA 95131, U.S.A.

Received February 18, 2019; accepted May 24, 2019; advance publication released online June 14, 2019

Endothelial cell injury and apoptosis induced by oxidative stress serve important roles in many vascular diseases. The repair of endothelial cell vascular injury relies on the function of local endothelial progenitor cells (EPCs). Our previous study indicated that epimedin C, a major flavonoid derived from Herba epimedii (yin yang huo), could promote vascularization by inducing endothelial-like differentiation of mesenchymal stem cells C3H/10T1/2 both in vivo and in vitro. In view of the significant cardiovascular protective effects of Herba epimedii, we detected a protective effect of epimedin C on hydrogen peroxide (H₂O₂)-induced peroxidation injury in human umbilical vein endothelial cells (HUVECs) and the role of EPC in this process. The results show that epimedin C increased the expression of the stem cell marker, CD34 and PROM1, and subsequently enhanced the expression and function of vascular endothelial growth factor and matrix metalloproteinase (MMP)-2 in local vascular endothelial cells. In conclusion, epimedin C protects H₂O₂-induced peroxidation injury by enhancing the function of endothelial progenitor HUVEC populations.

**Key words** epimedin C; endothelial progenitor cell; human umbilical vein endothelial cell; peroxidation injury; Herba epimedii; Chinese herb

**INTRODUCTION**

Endothelial cells are located in the interior lining of blood and lymphatic vessels throughout the human body. The endothelium, composed of a one-cell-thick layer of endothelial cells, provides not only a barrier between blood and tissue, but also a selectively permeable membrane across which fluids can travel.1 As endothelial cells are essential for angiogenesis, and function as an important gateway in managing inflammation, regulating blood clotting and blood pressure, and producing nitric oxide (NO), damage to these cells has become an important field of study in relation to aging, atherosclerosis and cardiovascular disease.2–4 Damage to endothelial cells and the endothelium can occur through inflammation caused by bacterial or viral infections, oxidative stress through dysregulation of reactive oxygen species (ROS), environmental factors, and as a result of hyperlipidemia. This damage can take the form of cellular apoptosis, or simply as dysregulated or decreased cell function.5

Herba epimedii (Epimedium brevicornu Maxim), also called yin yang huo, is a plant used in traditional Chinese medicine, primarily as an aphrodisiac and for strengthening bone, but also to reduce pain and numbness related to hemiplegia and general inflammation. It is traditionally believed to promote circulation as a vasodilator, to reduce inflammation, and, through its antihypertensive, antiarrhythmic and antihyperlipidemic properties, act as a cardiotonic.6 Herba epimedii has been shown to improve bone health, regulate hormone levels, modulate immunological functions, and inhibit tumor growth, as well as to act as an antidepressant and a neuroprotective agent.7

A primary constituent of Herba epimedii is epimedin C, a major flavonoid isolated from epimedium species, and recognized as a chemical marker for quality control for yin yang huo in China. Over 270 constituents have been identified from epimedium species, and approx. 115 metabolites in biosamples of epimedin C.8 Studies have indicated that epimedin C is metabolized via desugaramization, dehydrogenation, hydrogenation, dehydroxylation, hydroxylation, demethylation and glucuronidation pathways in vivo.9 Upon oral administration of epimedium, most flavanoids are hydrolyzed to secondary glycosides or aglycon by intestinal enzymes. There are two main sources of intestinal enzymes, intestinal mucosa and intestinal bacteria. Studies have demonstrated that the main flavanoids in epimedium could not be hydrolyzed by gastric juice, and, therefore, cannot be metabolized in the stomach.10 Thus, the main absorption site of epimedium flavanoids is the small intestine.11

It was previously demonstrated that epimedin C could induce endothelial-like cell differentiation through vascularization.12 We found that epimedin C enhanced bone morphogenetic protein 2 (BMP2) expression, induced osteogenesis of C3H/10T1/2 cells in BALB/c nude mice, but did not increase BMP2-dependent or -independent cell proliferation or alkaline phosphatase (ALP) activity in C3H/10T1/2 cells in vitro. In another study, PCR results indicated that the mRNA expression levels of classical endothelial markers, including CD34, Vezf1, Ang1 and Ang2, were significantly increased in C3H/10T1/2 cells after being treated with epimedin C for 5 d. The protein expression levels of CD31, CD73 and ESM-1 were also positively expressed after being treated with epimedin C for 5 d. Therefore, epimedin C may induce C3H/10T1/2 cells to differentiate into endothelioid cells.13

The present study aimed to demonstrate how epimedin C...
may protect hydrogen peroxide (H$_2$O$_2$)-induced peroxidation injury by enhancing the function of endothelial progenitor, and by increasing the expression of Prominin 1 (PRO1) vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-2 in local endothelial cells. The results show that epimedin C protects H$_2$O$_2$-induced cell apoptosis, and subsequently enhancing the function of endothelial progenitor human umbilical vein endothelial cell (HUVEC) populations (CD34$^+$), such as improving the activity or expression of VEGF and MMP-2 in local vascular endothelial cells.

MATERIALS AND METHODS

Materials  Epimedin C (Lot: 13112821) was purchased from Tauto Biotech (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) and kept at $\sim$20°C. was supplied by Sigma (St. Louis, MO, U.S.A.), phosphate buffered saline (PBS) powder, 0.25% (w/v) trypsin/1 mM ethylenediaminetetraacetic acid (EDTA), 30% H$_2$O$_2$ and 4% paraformaldehyde were purchased from the Hua dong Medicine Group Co., Ltd. (Hangzhou, Zhejiang, P. R. China). Vitamin C was purchased from Jiangxi New Ganjiang Pharmaceutical Co., Ltd. (Jiangxi, P. R. China). Diovan (Valsartan) was produced by Novartis Co., Ltd. (Switzerland).

Cell Culture and Treatments  HUVEC line was immortalized as described. Briefly, for immortalization of HUVECs, normal HUVECs cultured in Roswell Park Memorial Institute-1640 (RPMI 1640) (Gibco, Thermo Fisher Scientific, Inc.) medium supplemented with 20% fetal calf serum were transfected with the recombinant retrovirus (produced with pLXSN-SV40 LT vector) for 48h. Subsequently the transfected HUVECs were selected with 500 µg/mL G418 and 4 µg/mL puromycin at 72h after transfection for 14d. Drug resistant cells were selected and expanded for further studies.

For cell culture, HUVECs were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences, Logan, UT, U.S.A.), 2 mmol/L l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated in a humidified incubator with 5% CO$_2$ at 37°C with media replenishment every 2d and were passaged at 80–90% confluence.

The cells were divided into the following five groups: A, Normal group; B, H$_2$O$_2$ group; C, Epimedin C (1 µM) group + H$_2$O$_2$; D, Epimedin C (10 µM) group + H$_2$O$_2$; E, Epimedin C (100 µM) group + H$_2$O$_2$.

Investigation of Epimedin C and H$_2$O$_2$ Concentration  HUVECs were cultured in a 96-well plate (1 × 10$^5$ cells/well) for 24h. The medium with the final concentration of 100, 200, 400, 500, 800, 1000 and 4000 µM H$_2$O$_2$ or 0.1–300 µM epimedin C were added, each concentration was set to 3 wells. At the same time, the normal group was set and was incubated at 37°C for 12h (epimedin C) or 6–24h (H$_2$O$_2$). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the most appropriate damage concentration of H$_2$O$_2$ in HUVECs. The medium with 500 µM H$_2$O$_2$ was used to induced peroxidation injury.

Measurement of Lactate Dehydrogenase (LDH) Activity in Cell Supernatant  HUVECs were cultured in a 24-well plate (5 × 10$^4$ cells/well). After 12h plating, cells were intervened with 500 µM H$_2$O$_2$ for 20min, and 1, 10, 100 µM epimedin C and 50 µg/mL Vitamin C were added into the plate and cultured at 37°C for a further 12h. The LDH activity of cell supernatant were assayed with LDH activity assay kit (20170, Sangon Biotech, Shanghai, China) and detected by Synergy H1MFD multi-mode microplate reader (BioTek, Winooski, U.S.A.).

Apoptosis Analysis by Fluorescence Staining  Apoptosis cell was observed by fluorescence staining. HUVECs were cultured in a 6-well plate (3 × 10$^5$ cells/well). A total of 12h following plating, cells were intervened with 500 µM H$_2$O$_2$ for 20min, following which 1, 10, 100 µM epimedin C was added into the plate and cultured at 37°C for a further 12h. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was carried out with TUNEL Apoptosis Assay Kit (E607172, Sangon Biotech, Shanghai, China), cell were add 100 µL/well of 4% formaldehyde fixative buffer to each well and incubate plates for 20 to 30min at room temperature. And then remove fixative and wash the cell with PBS 3 times. Add 50 µL of the reaction mixture to each well and incubate at 37°C for 60min. Remove the reaction mixture, and wash the cells 5 times with 200 µL/ well of PBS. Hoechst assay was carried out by the Hoechst 33258 assay kit (C1017, Beyotime Biotechnology, Shanghai, China). Finally, the red DNA fragments staining and blue nuclei was examined under fluorescence microscopy (Nikon Ti-S, Tokyo, Japan).

Apoptosis Analysis by Flow Cytometry  Apoptosis was evaluated by using the Cell Apoptosis analysis kit (556547, FITC Annexin V Apoptosis Detection Kit 1, BD Pharmingen, U.S.A.). Briefly, HUVECs were cultured in a 6-well plate (3 × 10$^5$ cells/well) and treated with 500 µM H$_2$O$_2$ and/or epimedin C for 12h. Cells were harvested by trypsin and washed with PBS. Cells were resuspended with 150 µL of 1 × binding buffer, and were incubated with 5 µL fluorescein isothiocyanate (FITC) at room temperature for 15min and 5 µL propidium iodide (PI) for 5min. 100 µL of 1 × binding buffer was added and the cells were detected by flow cytometry (Guava EasyCyte 6HT2L, Merck KGaA, Darmstadt, Germany). Apoptotic cells and death cells were detected, apoptotic cells were calculated and expressed at a percentage.

Cell Immunofluorescence  HUVECs were cultured in a 6-well plate (3 × 10$^5$ cells/well). A total of 12h following plating, cells were intervened with 500 µM H$_2$O$_2$ for 20min, following which 1, 10, 100 µM epimedin C was added into the plate and cultured at 37°C for a further 12h.After treatment, the cells were washed with PBS, fixed with 10% paraformaldehyde for 10min, incubated with 0.5% triton solution for 15min in the dark, blocked with 5% BSA for 20min. The cells were incubated overnight at 4°C with primary antibody (sc-7269, anti-VEGF (C-1), Santa, U.S.A., 1:100 or 553733, FITC Rat Anti-Mouse CD34, BD Pharmingen, U.S.A., 1:100). Cells labeled with VEGF antibody were followed by incubation with a secondary antibody (sc-2781, Goat Anti-Mouse immunoglobulin G (IgG)-TR, Santa, U.S.A., 1:200) for 2h at room temperature. Finally, the cells were stained with 0.5 µg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 15min and sealed with 50% glycerol. Pictures were taken with Fluorescence microscopy (EVOS FL, American thermoelectric, American). Blue fluorescence was DAPI-labeled nuclei; red fluorescence was the target protein expression of VEGF; green fluorescence was the target protein expression of CD34.

Real-Time Quantitative PCR (qPCR)  HUVECs were
intervened with 500 µM H₂O₂ for 20 min, following with 1, 10, 100 µM Epimedin C for 24 h. The total RNA of all cardiomyocytes was extracted with Trizol (Invitrogen), purity and concentration of the extracted RNA were measured on Trace nucleic acid protein detector (Nanodrop 2000, ThermoFisher, U.S.A.). Then cDNA was synthesized by reverse transcription, and fluorescence quantitative detection of the target gene was performed afterwards. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. All reactions were performed in a thermal cycler (ABI7500, ABI, U.S.A.) with primers shown in Table 1 (Sangon Biotech, Shanghai, China).

For GAPDH, forward: 5'-CACG CGAC ACCCA CTCC TCC-3' and reverse: 5'-TGAGT TCCA CACC CACCC TGT-3'; reduced nicotinamide adenine dinucleotide phosphate (NADPH), forward: 5'-GGAGAGCCATGAGCA GTC ATC C-3' and reverse: 5'-GATT GCTG TGTG TGC-3'; thioredoxin reductase (TR), forward: 5'-AGG AACGG ATGAA CAG GC-3' and reverse: 5'-AGATG GACGCC TCTGT TGT-3'; Heme oxygenase-1 (HO-1), forward: 5'-AAC TTCT CAGA AGG GCCG AG-3' and reverse: 5'-AGA CTGG CTCGCC TCTGT TGT-3'; Peroxiredoxin-6 (Prx6), forward: 5'-CGT CTG GATG AAGG CGA CAT-3' and reverse: 5'-GTTG GATG ACCA GTC ATC C-3'; Peroxiredoxin-5 (Prx5), forward: 5'-ATGTG CAGG ATGAA CAG GC-3' and reverse: 5'-ACAA GGGAC TACTG TCA-3'; Peroxiredoxin-2 (Prx2), forward: 5'-CGTCT GGTG GAGT GTA GGT-3' and reverse: 5'-CCAAG AGATG TCTG TCA-3'; Oxiredoxin-2 (Prx2), forward: 5'-GAC GAA CCCA AAGG CAC-3' and reverse: 5'-GTA AGTGG ACCA GAG GC-3'; TIMP2, forward: 5'-CTCTT CGTGT TCTG AGA GTC-3' and reverse: 5'-GAA CTGG CATGCC ATC AAAT-3'; MMP9, forward: 5'-GGAG GGCA CGA CAG-3' and reverse: 5'-TTGTT CAGGG GGA CTG-3'; MMP2, forward: 5'-TGATG AGTGA GAGT GTC-3' and reverse: 5'-GAGT GATG ACCA GAG GC-3'; TIMP1, forward: 5'-ATGTG CAGG ATGAA CAG GC-3' and reverse: 5'-GTA AGTGG ACCA GAG GC-3'; KDR, forward: 5'-TGAGG GAATCT GGA ACCGC-3' and reverse: 5'-GTTG GATG ACCA GAG GC-3'; CD34, forward: 5'-CGTCT GGT GGA TGC CAG-3' and reverse: 5'-GGAAG GCCG AGT TAC-3'; PROM1, forward: 5'-GGG ACCG TCTG TGT-3' and reverse: 5'-CTCTG TCTG GTG GAC-3'.

Table 1. PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’ to 3’)</th>
<th>Primers (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CACGCA CACCCA CTCCTC</td>
<td>TGAAGT CACCCA CTCCTC</td>
</tr>
<tr>
<td>NADPH</td>
<td>GGAAGCATGAGCA GACCTC</td>
<td>AATGTTCA GATGGT CAC</td>
</tr>
<tr>
<td>TR</td>
<td>AGGAA CGATGGAA CTCAGG</td>
<td>GTGAA ATGGT CACG</td>
</tr>
<tr>
<td>HO-1</td>
<td>AACTTT CGAGAGGCCC AGG</td>
<td>AGACTGGG CTCTCTTGT</td>
</tr>
<tr>
<td>Prx5</td>
<td>GATGTCGCTGTTACCATCT</td>
<td>CTGTCGCATGTT CAC</td>
</tr>
<tr>
<td>Prx6</td>
<td>CAGTGTGCA CCAAGAGCAGGTT</td>
<td>GGCAG ATGTTGCCA CAC</td>
</tr>
<tr>
<td>MMP2</td>
<td>CTTCCCTGCTGCCAGA GAGT</td>
<td>CAGTGG CATGCC ATC AAAT</td>
</tr>
<tr>
<td>MMP9</td>
<td>CCCGCA CACAGATGACGT</td>
<td>GCCATTCACGCCGAT TAC</td>
</tr>
<tr>
<td>TIMP1</td>
<td>GGGGCTTCCA CCAAGACTA</td>
<td>AAGTGGAC GAGGACTGGA GAG</td>
</tr>
<tr>
<td>TIMP2</td>
<td>AGGAATCGGTGAGTT CCTG</td>
<td>ACA CAA GCCGGAT AAAGC</td>
</tr>
<tr>
<td>CD34</td>
<td>GAGAAAGGCGC VGGGCG GAAAGGGCAT</td>
<td>GTGTCGTGCTGCA AAAT GCCG</td>
</tr>
<tr>
<td>CD31</td>
<td>GGGTACGTTGAGGACGAGGAGA CAT</td>
<td>GGTGATG GACCAGATCT CTC</td>
</tr>
<tr>
<td>KDR</td>
<td>GAGGGAAGACCAGATGAGGAGGACGG</td>
<td>GGC CAAAGGCTGTTACTCAGC</td>
</tr>
<tr>
<td>PROM1</td>
<td>GTCTG GGGCTGTGCTTAT</td>
<td>TCTGTCGCTGGT GCATTCT</td>
</tr>
</tbody>
</table>

RESULTS

Epimedin C Reduced LDH Activity in H₂O₂ Treated HUVECs

The results of MTT assays revealed that epimedin C (0–100 µM) had no effect on cell proliferation and slightly promoted cell proliferation at 1–30 µM (Fig. 1A). The study showed that 1, 10, 100 µM epimedin C for 12 h was the most appropriate concentration and duration for repair.

The MTT assay for H₂O₂ shown that cell viability decreased as the concentration of H₂O₂ increased (Supplementary Figure 1A). When the concentration of H₂O₂ reached 500 µM, the cell survival rate decreased to 50%, indicating cell injury. Subsequently, we compared the influence of 500 µM H₂O₂ on HUVECs in different time points, the cell mortality was >50% at 6 and 24 h. Taking this into account, 500 µM H₂O₂ for 20 min was selected for the following experiment.

LDH activity of HUVECs was increased by H₂O₂ treatment. As shown in Fig. 1C, the LDH activity in H₂O₂ group
Epimedin C Protected Oxidative Damage of DNA Induced by H$_2$O$_2$ The apoptotic cells in the nucleus or cytoplasm appeared dense and granular by fluorescence microscopy (Nikon Ti-s, Nikon). TUNEL positive cells was calculated under a fluorescent microscope. Furthermore, chromatin shrinkage and DNA damage were evident. Compared with the H$_2$O$_2$ group, the proportion of normal cells in the epimedin C (1, 10 and 100 µM) + H$_2$O$_2$ groups were significantly increased, and protected against DNA damage. This effect was dose-dependent (Fig. 2).

Epimedin C Reduced H$_2$O$_2$-Induced Apoptosis HUVECs The results show that the apoptotic rate was 2.41 ± 0.21% in the normal group and 64.49 ± 0.48% in the control group (p < 0.01); while the early apoptosis rate of the epimedin C (100 µM) + H$_2$O$_2$ group decreased to (50.41 ± 1.38%), which was significantly different compared with the control group (p < 0.01) (Fig. 3). However, compared with the control group, the apoptotic rate of the epimedin C (1 and 10 µM) + H$_2$O$_2$ group increased. As a result, epimedin C increased the proportion of living cells, but induced cells apoptosis moved to late-apoptosis or dead.

Epimedin C Increased the mRNA Expression Levels of HO-1, Prx2, Prx6 and TR in H$_2$O$_2$-Treated HUVECs The peroxidative injury of human umbilical vein endothelial cells is related to the expression of NADPH, Prx2, Prx6 and TR. The expression of NADPH, Prx2, Prx6 and TR were significantly down-regulated (p < 0.05) in control group. As shown in Fig. 4, after treatment with 1 µM epimedin C + H$_2$O$_2$, the mRNA levels of TR and Prx6 were significantly up-regulated (p < 0.05). After treatment with 10 µM epimedin C + H$_2$O$_2$, the expression of HO-1 was up-regulated (p < 0.01), the mRNA level of NADPH was also up-regulated but without statistical difference. In a word, the epimedin C can repair the peroxidation-injured of human umbilical vein cells, which is related to the up-regulation of the mRNA levels of related enzymes such as HO-1, TR, Prx2 and Prx6.

Epimedin C Increased the Expression of VEGF and CD34 Protein in HUVECs As shown in Fig. 5, positive VEGF and CD34 staining was detected by IF. CD34 localized predominantly along cell membrane (green fluorescence in Fig. 5) and VEGF (red fluorescence in Fig. 5) mainly in the cytoplasm. It could be observed from the results that the expression levels of VEGF and CD34 protein were decreased in the control group, and increased in the epimedin C + H$_2$O$_2$ group.

Epimedin C Improved MMP2 and MMP9 Activity Inhibited by H$_2$O$_2$ in HUVECs The effect of epimedin C on
MMP2 and MMP9 activation was analyzed by gelatin zymography. As shown in Fig. 6E, MMP2 and MMP9 secretion was significantly reduced by H$_2$O$_2$ treatment and slightly increased by epimedin C after 12 h treatment. The results suggest that epimedin C can improve the relative activity of MMPs, which is suppressed by peroxidation injury. However, the mRNA levels of TIMP1 and TIMP2 were significantly reduced by H$_2$O$_2$ treatment and increased by 10$\mu$M epimedin C treatment for 48 h (Figs. 6C, D). It suggests that the influence of epimedin C on the expression of MMPs may decrease first and then increase.

DISCUSSION

Peroxidation injury is caused by the imbalance between the systemic manifestation of reactive oxygen species and detoxification ability. The production of peroxides and free radicals can damage all components of the cell, including proteins, lipids and DNA. The base damage caused by ROS, including O$_2^-$ (superoxide radical), OH (hydroxyl radical) and H$_2$O$_2$ (hydrogen peroxide), can induce strand breaks in DNA. Peroxidation injury is thought to be involved in the development of Asperger syndrome, cancer, Parkinson’s disease, Alzheimer’s disease, atherosclerosis, heart failure, myocardial infarction and depression. Therefore, repairing the resulting damage is a central theme of medical research.

Our results demonstrate that epimedin C may exert a protective effect against H$_2$O$_2$-induced peroxidation injury in HUVECs. This may be explained by epimedin C enhancing antioxidative defense. Epimedin C increased the mRNA expression levels of HO-1 (a rate-limiting enzyme that catalyzes oxidative degradation of cellular heme to liberate free iron), Prx2 and 6 (antioxidant enzymes) and TR (a central component in the thioredoxin system) which decreased by H$_2$O$_2$-induced peroxidation injury in HUVECs.

The repair of damaged tissue is dependent on the function of local EPCs (CD34$^+$). These stem cells serve two important roles in tissue repair: Differentiating into new cells to replace damaged tissue (tissue-specific resident stem cells) or aiding in the regenerative or reparative process via mesenchymal stromal cells.$^{15}$ Numerous other studies have suggested that endothelial progenitor cells can differentiate into endothelial
cells, and restore some endothelium function. Beginning with the landmark work of Asahara, it has been shown that bone marrow-derived EPCs participate in normal and pathological vessel formation in adults, which was previously thought to only occur in utero. Salter and Sehmi revealed that EPCs can differentiate locally within tissue into ECs, contributing to vascular repair, maintenance and expansion under pathological conditions.

Although the underlying mechanism remains unclear, VEGF is an essential factor in angiogenesis, a vital process.

Fig. 3. The Effects of Epimedin C on the Apoptotic Rate of HUVECs Induced by H$_2$O$_2$
Data are from three independent experiments. Values are presented as the mean ± S.D. (n = 3). *p < 0.05 and **p < 0.01 vs. Normal group, *p < 0.05 and **p < 0.01 vs. Control group. (A) the normal group, (B) the Control group, (C) the epimedin C (100µM) + H$_2$O$_2$ group and (D) cell apoptotic rate.

Fig. 4. The Relative Expression of NADPH, HO-1, Prx2, Prx6, and TR mRNA (A–E)
Data are from three independent qPCR experiments. Values are presented as the mean ± S.D. (n = 3). *p < 0.05 and **p < 0.01 vs. Normal group, *p < 0.05 and **p < 0.01 vs. Control group.
not only for growth and development, but also in the healing of tissue.\textsuperscript{18} The present study indicates that epimedin C may increase the level of VEGF expressed in the cell cytoplasm and membranes, particularly in CD34-positive HUVECs. This, in turn, may lengthen the life span of endothelial cells and prevent apoptosis. This effect is accomplished by inducing the transient expression of anti-apoptotic proteins.\textsuperscript{19} Further research is required to determine the optimal dose of epimedin C, its relationship with VEGF, and the optimal conditions for administration.

Similarly, to VEGF, epimedin C may also serve a role in the protection and upregulation of CD34 expression. While little is known about its exact function, CD34 serves an essential role in cell recruitment and migration during tissue
healing and is an important marker of stem cell activity. It has been demonstrated that epimedin C increased the expression of CD34 and VEGF. CD34 hematopoietic stem cells have been used clinically to treat spinal cord injuries, liver cirrhosis and peripheral vascular disease. Potential future studies could include oral administration of epimedin C during injectable stem cell treatments, or pretreatment of stem cell injectables with epimedin C to increase the effectiveness of such protocols.

Matrix metalloproteinases (MMPs) are a group of enzymes produced throughout the body, which are essential in normal physiological processes. It is known that MMPs alter the ECM, leading to the disintegration of tissue integrity and the infiltration of neutrophils and macrophages. It has been suggested that MMP-9 participates in paracetamol-induced hepatotoxicity mediated by sinusoidal endothelial cell injury, which results in the impairment of microcirculation. In this study, the protein expression of MMP-2 and MMP-9 was inhibited by H$_2$O$_2$ in HUVECs, and epimedin C enhanced the activity of MMP-2 but not MMP-9.

CONCLUSION

The present study described the protective effect of epimedin C on H$_2$O$_2$-induced peroxidation injury in HUVECs. Epimedin C was demonstrated to protect cell injury via increasing the number and reinforcing the activity of EPCs. Briefly, epimedin C induces VEGF and MMP-2 expression and function in HUVECs, as well as the expression of the endothelial stem cell marker, CD34. In addition, our earlier study demonstrated that epimedin C induced C3H/10T1/2 cells to differentiate into vascular endothelial cells both in vivo and in vitro. Although the mechanism remains unclear, a series of studies from our research group, including this study, have demonstrated the value of epimedin C in cardiovascular protection.

Acknowledgments This work was supported by the funds from the Medical and Health General Research Plan of Zhejiang Province (No.2017KY068 to DH Wei), the National Natural Science Foundation of China (81473575 to JL Gao), and the Young Talent Cultivation Project of Zhejiang Association for Science and Technology (Grant No. 2016YCGC002 to JL Gao).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


