Epimedin C protects H₂O₂-induced peroxidation injury by enhancing the function of endothelial progenitor HUVEC populations

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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 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 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; HUVEC; peroxidation injury; Herba Epimdii; Chinese herbs
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 ~115 metabolites in biosamples of epimedin C\(^8\). Studies have indicated that epimedin C is metabolized via desugarization, dehydrogenation, hydrogenation, dehydroxylation, hydroxylation, demethylation and glucuronidation pathways \textit{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 flavonoids 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 flavonoids 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 days. The protein expression levels of CD31, CD73 and ESM-1 were also positively expressed after being treated with epimedin C for 5 days. 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 PROM1, VEGF and 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 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 DMSO and kept at -20°C. Dimethyl sulfoxide (DMSO) was supplied by Sigma (St. Louis, MO, USA), phosphate buffered saline (PBS) powder, 0.25% (w/v) trypsin/1 mM EDTA, 30% H\(_2\)O\(_2\) and 4% paraformaldehyde were purchased from the Huadong 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. (Swiss).
**Cell culture and treatments** Human umbilical vein endothelial cell line (HUVEC) was immortalized as described\(^{14}\). 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 48 h. Subsequently the transfected HUVECs were selected with 500 μg/mL G418 and 4 μg/mL puromycin at 72 h after transfection for 14 d. 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, USA), 2mmol/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 2 days 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\(^4\) 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\(_2\)). 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 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 20 min, and 1 μM, 10 μM, 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 (201710, Sangon Biotech, Shanghai, China) and detected by Synergy H1MFD multi-mode microplate reader (BioTek, Winooski, USA).
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 500μM H_2O_2 for 20 min, following which 1 μM, 10 μM, 100 μM epimedinc C was added into the plate and cultured at 37°C for a further 12 h. 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 30 min 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 60 minutes. 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, USA). Briefly, HUVECs were cultured in a 6-well plate (3×10^5 cells/well) and treated with 500 μM H_2O_2 and/or epimedin C for 12h. Cells were harvested by trypsin and washed with PBS. Cells were re-suspended 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_2O_2 for 20 min, following which 1 μM, 10 μM, 100 μM Epimedin C were 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, USA, 1: 100 or 553733, FITC Rat Anti-Mouse CD34, BD Pharmingen, USA, 1: 100). Cells labeled with VEGF antibody were followed by incubation with a secondary antibody (sc-2781, Goat Anti-Mouse IgG-TR, Santa, USA, 1: 200) for 2h at room temperature. Finally, the cells were stained with 0.5μg/mL 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 μM, 10 μM, 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, USA). Then cDNA was synthesized by reverse transcription, and fluorescence quantitative detection of the target gene was performed afterwards. GADPH was used as an internal control. All reactions were performed in a thermal cycler (ABI7500, ABI, USA) with primers shown in Table 1 (Sangon Biotech, Shanghai, China).

for GADPH (glyceraldehyde-3-phosphate dehydrogenase), forward: 5’- CAG CGA CAC CCA CTC CTC-3’ and reverse: 5’- TGA GGT CCA CCA CCC TGT -3’; NADPH (Nicotinamide adenine dinucleotide phosphate), forward: 5’- GGA AGC CAT GAG CAA GTC TC -3’ and reverse: 5’- AAT GTT CCA GTT GGG TCC AG-3’; TR (thioredoxin reductase), forward: 5’- AGG AAC CGA GTC TCC AGT GA-3’ and reverse: 5’- GGT GAA GTC TGT GCT GTC CA-3’; HO-1 (Heme oxygenase-1), forward: 5’- AAC TTT CAG AAG GGC CAG GT-3’ and reverse: 5’- AGA CTG GGC TCT CCT TGT TG-3’; Prx2 (Peroxiredoxin-2), forward: 5’- CGT CTC GGT GGA CTC TCA GT-3’ and reverse: 5’- TCA GAC AAG CGT CTG GCC GTC AC-3’; Prx5 (Peroxiredoxin-5), forward: 5’- GAT TCG CTG GTG TCC TTC TT-3’ and reverse: 5’- CTG TGC CAT CTG GTT CCA CG-3’; Prx6 (Peroxiredoxin-6), forward: 5’- CAG TGT GCA CCA CAG AGC TT-3’ and reverse: 5’-GGC AAG ATG GTC CTC AAC AC -3’. The results of qPCR were analyzed, that is, the Ct values
of each sample were analyzed, and the $2^{-\Delta\Delta CT}$ values of each target gene were calculated, and the differences in the expression levels of different groups of HUVECs cells were obtained.

Table 1. PCR primers

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<th>Gene</th>
<th>Primers (5' to 3')</th>
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**Gelatin zymography analysis** Gelatin zymography was used to assess the extent of MMP2 and MMP9 activity. HUVECs were cultured in a 24-well plate (5×10⁴ cells/well). A total of 12h following plating, cells were intervened with 500 µM H₂O₂ for 20 min, following which 1 µM, 10 µM, 100 µM epimedin C was added into the plate and cultured at 37°C for a further 12h. Then cell supernatant was harvested and mixed with Tris-Glycine SDS Sample Buffer (1x) (1:1, v/v) and let stand for 10 min at room temperature. After normalizing the total protein aliquots of the samples, the samples were subjected to SDS-PAGE in a 10% gel impregnated with 0.1 mg/mL gelatin. After electrophoresis, the gels were incubated in 25% Triton X-100 for 30 min at 37°C to remove SDS and in an incubation buffer (50 mM Tris-HCl buffer, pH 7.8, containing 5 mM CaCl₂, 0.15 mM NaCl, 0.02%NaN₃) for 42h at 37°C. Then, the gels were stained with Coomassie brilliant blue R-250 for 3h. After that, gels were de-stained with an appropriate Coomassie R-250 de-staining solution (Methanol : Acetic acid : Water =50 : 10 : 40, v/v/v). Gelatinolytic activity was detected as unstained bands on a
blue background. The position of bands was identified on the basis of the molecular weight protein standard (BioRad).

**Statistical analysis** Data was analyzed by using Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Data was expressed as means ± standard deviation (SD). The significance of the difference among mean values was evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. The difference between mean values was determined by student’s t test. P< 0.05 were considered to be statistically significant.

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 1μM, 10μM, 100μM 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 (sup. Fig. 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 h and 24 h. Taking this into account, 500 μM H₂O₂ for 20 min was selected for the following experiment.

Lactate dehydrogenase (LDH) activity of HUVECs was increased by H₂O₂ treatment. As shown in Fig. 1C, the LDH activity in H₂O₂ group was significantly higher (P<0.01) than that of the normal group and Vc can significantly reduce the LDH activity (P<0.01). After intervention with epimedin C, LDH activity were decreased. LDH activity of the epimedin C groups was significantly decreased (P<0.01, respectively).
Fig. 1. (A) The structure of epimedin C. (B) The effects of different concentrations of epimedin C treatment on cell activity \((n=3)\). Data are from three independent experiments. (C) The effects of epimedin C on LDH activity of HUVECs induced by H\(_2\)O\(_2\). Data are from three independent experiments. Values are presented as the mean ± SD \((n=3)\). \(^{#}P<0.05\) and \(^{##}P<0.01\) vs. Normal group, \(^{*}P<0.05\) and \(^{**}P<0.01\) vs. Control 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, Japan). 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 \mu M, 10 \mu M \text{ and } 100 \mu M) + H\(_2\)O\(_2\)\) groups were significantly increased, and protected against DNA damage. This effect was dose-dependent (Fig. 2).
**Fig. 2.** The effects of epimedin C on the morphological changes of HUVECs induced by H$_2$O$_2$. A-C: Hoechst 33258 assay, (A) Normal group, (B) Control group, (C) Epimedin C (10 µM) + H$_2$O$_2$ group, D-F: TUNEL Apoptosis Assay, (D) Normal group, (E) Control group, and (F) Epimedin C (10 µM) + H$_2$O$_2$ group. Photographs were made under a microscope at a magnification of 200X. The results represent three independent experiments. (G) the quantification of TUNEL assay.

(Color figure can be accessed in the online version.)

**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 µM and 10 µM) + H2O2 group increased. As a result, epimedin C increased the proportion of living cells, but induced cells apoptosis moved to late-apoptosis or dead.

Fig. 3. The effects of epimedin C on the apoptotic rate of HUVECs induced by H2O2. Data are from three independent experiments. Values are presented as the mean ± SD (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 (100 µM) + H2O2 group and (D) cell apoptotic rate.

**Epimedin C increased the mRNA expression levels of HO-1, Prx2, Prx6 and TR in H2O2-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+H2O2, the mRNA levels of TR and Prx6 were significantly up-regulated (P<0.05). After treatment with 10 μM epimedin C+H2O2, 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.

**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 ± SD ($n=3$). 

\* $P<0.05$ and \#\# $P<0.01$ vs. Normal group, \* \* $P<0.05$ and \** $P<0.01$ vs. Control group.

**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 figure 5) and VEGF (red fluorescence in figure 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.
Fig. 5. (A) The effects of epimedin C on VEGF and CD34 expression in HUVECs induced by H2O2. DAPI, CD34 and VEGF and merge images of the Normal group, the Control group and the epimedin C (1μM) + H2O2 group. Photographs were made under a microscope at a magnification of 400X. (B-E) The relative mRNA expression of CD31, CD34, PROM1, and KDR. Data are from three independent qPCR experiments. Values are presented as the mean.
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 µM epimedin C treatment for 48h (Figure 6C and D). It suggests that the influence of epimedin C on the expression of MMPs may decrease first and then increase.

(Fig. 6. (A-D) The relative mRNA expression of MMP2, MMP9, TIMP1 and TIMP2. Data are from three independent qPCR experiments. Values are presented as the mean ± SD (n= 3). #P<0.05 and ##P<0.01 vs. Normal group, *P<0.05 and **P<0.01 vs. Control group. (F) The effects of epimedin C on MMP2 and MMP9 secretion by HUVECs induced by H$_2$O$_2$. )
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 $^{16}$. Salter and Sehmi revealed that EPCs can differentiate locally within tissue into ECs, contributing to vascular repair, maintenance and expansion under pathological conditions $^{17}$.

Although the underlying mechanism remains unclear, VEGF is an essential factor in angiogenesis, a vital process 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\textsuperscript{20}, 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\textsuperscript{21}, liver cirrhosis\textsuperscript{22} and peripheral vascular disease\textsuperscript{23}. 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\textsuperscript{24}. 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\textsuperscript{25}. In this study, the protein expression of MMP-2 and MMP-9 was inhibited by H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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.

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**Conflict of interest** The authors declare no conflict of interest.

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


