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 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.2–5) 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.6)

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.7) 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.8)

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 in 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 desugarization, dehydrogenation, hydroxylation, dehydroxylation, hydroxylation, demethylation and glucuronidation pathways in vivo.9) Upon oral administration of epimedium, most flavonoids 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 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 (PROM1) 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 −20°C. was supplied by Sigma (St. Louis, MO, U.S.A.), phosphate buffered saline (PBS) powder, 0.25% (w/v) trypsin/1mM ethylenediaminetetraacetic acid (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). Diovon (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 4µg/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.), 2mmol/L l-glutamine, 100U/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 d 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$_2$). 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 HI1MFD multi-mode microplate reader (BioTek, Winooski, U.S.A.).

Apoptosis Analysis by Flow Cytometry

Apoptosis was observed by fluorescence staining. HUVECs were cultured in a 6-well plate (3 × 10$^4$ 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 60 min. 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$^4$ 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 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$^4$ 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% trition 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

**Table 1. PCR Primers**

| Gene                  | F                  | R                  |
|-----------------------|--------------------|--------------------|
| GAPDH                 | CAGCGA CACCCA CTCCTC | TGGAGGT CCA CCA CCCC TGT |
| NADPH                 | GAGAGC CATG AGAAGCTC | AATGTTCCA GTA GGGTCCAG |
| TR                    | AGGAACCG CATG CTCC AGTGA | GTTGAAGCT TCTGCTGTCACA |
| HO-1                  | AACTTTC CAGA GGGC CAGT | AGACTGGGCT TCTCTGTTG |
| Prx5                  | GATTCGCTGTG TCTCAGTTT | GTCTGCGCAT CTGTTCCACA |
| Prx6                  | CAGTGTGCA CACAAGAGGCTT | GCCAGAATGTGCTCTAACAC |
| MMP2                  | CTCCTCTGCT TCCAGAGT | TACGAAGGCT GACCAT AAAT |
| MMP9                  | CGGA CGCA AAGGATA CAGT | GCCATTTCCAGT GTCCTTA |
| TIMP1                 | GGGCTCTCA CCA AGACTA | AAGTGCAGC CGGACTGAGG |
| TIMP2                 | AGGAATCGTG TGA GTTCTG | ACA CAA GCCG CGTATAGGC |
| CD34                  | GAGAGAACGGC TGGGAGGAC | GTGTGCTTGGT AATGGGCG |
| CD31                  | GTTGAAGTC GTGAGAGGACAT | GGTGATGAGG ACCA GCTCTGC |
| KDR                   | GAGGGG AAATG CAGA GAGGC | GCCCAAGAGGCTTAC CACG |
| PROM1                 | GTCCGT GGGG CTG TCATTTAT | TCTGCGCT GTGGCATTCT |

**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 of 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 was decreased. LDH activity of the epimedin C groups was significantly decreased \((p<0.01, \text{respectively})\).

**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 \((1, 10 \text{ and } 100 \mu M) + \text{H}_2\text{O}_2\) 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 \(2.41 \pm 0.21\%\) in the normal group and \(64.49 \pm 0.48\%\) in the control group \((p<0.01)\); while the early apoptosis rate of the epimedin C \((100 \mu M) + \text{H}_2\text{O}_2\) group decreased to \(50.41 \pm 1.38\%\), 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 \((1 \text{ and } 10 \mu M) + \text{H}_2\text{O}_2\) 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 \(1 \mu M\) epimedin C + \text{H}_2\text{O}_2,\) the mRNA levels of TR and Prx6 were significantly up-regulated \((p<0.05)\). After treatment with \(10 \mu M\) epimedin C + \text{H}_2\text{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 + \text{H}_2\text{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 H2O2 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 H2O2 treatment and increased by 10 µM 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 O2− (superoxide radical), OH (hydroxyl radical) and H2O2 (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 H2O2-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 H2O2-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. 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

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**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.

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**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

Fig. 5. (A) The Effects of Epimedin C on VEGF and CD34 Expression in HUVECs Induced by H\textsubscript{2}O\textsubscript{2}. DAPI, CD34 and VEGF and Merge Images of the Normal Group, the Control Group and the Epimedin C (1\textmu M) + H\textsubscript{2}O\textsubscript{2} Group; Photographs Were Made under a Microscope at a Magnification of 400 ×; (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 ± S.D. (n = 3). *p < 0.05 and **p < 0.01 vs. Normal group, *p < 0.05 and **p < 0.01 vs. Control group. (Color figure can be accessed in the online version.)
healing,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,21) liver cirrhosis22) and peripheral vascular disease.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.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.25) In this study, the protein expression of MMP-2 and MMP-9 was inhibited by H2O2 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 H2O2-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.

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