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Research Article

Synergic Effect of *Ligusticum chuanxiong* Hort Extract and Borneol in Protecting Brain Microvascular Endothelial Cells against Oxygen-Glucose Deprivation/Reperfusion Injury

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Abstract

Background and Objective: *Ligusticum chuanxiong* Hort (LC) and borneol (BO) are usually prescribed as a combination of ischemic stroke patients for a better therapeutic effect in China. However, their synergic mechanism is unclear. Considering the critical role of Brain Microvascular Endothelial Cells (BMECs) in this disorder, the present study was designed to explore their synergic mechanism in protecting BMECs against Oxygen-Glucose Deprivation/Reperfusion (OGD/R) injury. **Materials and Methods:** The primary cultured BMECs were identified and divided into 5 groups of control, model, ELC (50 mg L^{-1}), BO (50 μg L^{-1}) and ELC+BO. Then their synergic treatment was evaluated via measuring oxidative stress, $[Ca^{2+}]i$, apoptosis ratio, levels of apoptosis-related genes and angiogenesis-related proteins. **Results:** After a comparison between the combined group and their monotherapies, it was shown that the superiority of ELC was in inhibiting oxidative stress and apoptosis, regulating $[Ca^{2+}]i$ and levels of apoptosis-related genes, while that of BO was in promoting angiogenesis. Interestingly, the combined therapy even reduced VEGFR1 expression which was unaffected in both of their monotherapies. **Conclusion:** ELC and BO might play different roles in protecting BMECs against oxygen-glucose deprivation/reperfusion injury and their synergic effect was displayed in the present study.

Key words: *Ligusticum chuanxiong* Hort, borneol, brain microvascular endothelial cells, primary culture, synergic mechanism, angiogenesis, calcium overload

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stroke, as the second leading cause of death, usually induced severe long term disability worldwide, especially in the elderly population in which stroke incidence is the highest[1]. Ischemic stroke is the most common stroke in the clinic and always presented as ischemia/reperfusion injury of the brain. For several decades, the attempt to explore effective drugs against ischemic stroke via neuroprotection seems to fail[2]. Presently, neuronal scientists intensify their work on improvement of stroke recovery, so-called neuronal restorative therapy, which has a much longer therapeutic window than neuroprotection[3]. Because angiogenesis is an important part of the neuronal restorative event, its critical role in improving ischemic stroke attracts scientist's attention widely[2].

The brain vascular endothelial cell is susceptible to ischemic attack, which may reversely damage cerebral blood circulation and cause ischemia disorder to deteriorate further. Just because of this, brain microvascular endothelial cells (BMECs) is regarded as a key therapeutic target in ischemic stroke[4]. Both protecting BMECs and even promoting angiogenesis are important issues in ameliorating ischemic stroke.

Ligusticum chuanxiong Hort (LC), a member of Umbelliferae plants, is a very common ethnomedicine in Asia, including China, Japan and Korea and widely used for some cardio-cerebrovascular disease such as atherosclerosis, ischemic stroke, migraine and coronary heart disease with satisfied outcome[5-7]. Borneol (BO), a bicyclic monoterpene compound, has been frequently prescribed for CNS disorder diseases, including Alzheimer's disease, stroke, cerebritis, cerebral edema, insomnia and giddiness in China[8,9]. It has been verified that BO not only helps transportation of drugs across the Blood-Brain Barrier (BBB) to increase their deliveries in brain[10-12] but also attenuates cerebral ischemia by itself, including reductions of oxidative stress, inflammation reactions, infarction area and apoptosis[13-16].

In the clinic, it is common to prescribe the combination of LC and BO for ischemic stroke. In our previous research, their synergic effect in improving brain ischemia was confirmed and its mechanisms were involved in inhibiting oxidation, regulating apoptosis, autophagy and Ca^{2+} overload[17]. However, it is unclear whether their synergic mechanism is directly related to protecting BMECs.

Therefore, the present research was aimed to further explore their synergic mechanism on BMECs protection against Oxygen-Glucose Deprivation/Reperfusion (OGD/R) injury *in vitro*.

MATERIALS AND METHODS

The study was carried out at the Department of Pharmacy, Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, China from September 2019-January, 2020.

Materials: Both LC and BO were obtained from Nanjing Pharmaceutical Co., Ltd. (Nanjing, China). Saline solution and other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The preparation and identification of ELC were performed as our previous report[17].

Preparation of primary BMECs: BMECs were obtained from 24 hrs old neonatal SD rats as described previously[18]. Briefly, the rat brain was separated, cut into 1 mm^3, homogenized at 4°C and centrifuged at 600 g for 10 min. The deposition was suspended in DMEM/F12 and filtered. The BMECs retained on the screen mesh were collected and then digested with type-2 collagenase. Then the precipitate was suspended in DMEM with 20% bovine serum albumin (BSA) and centrifuged. The pellets were re-suspended and washed. The BMECs were maintained in DMEM/F12 medium, containing 20% FBS, 3 mg mL^{-1} glucose, 0.58 mg mL^{-1} L-glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at a density of 1×10^5 mL^{-1} and incubated at 37°C with 5% CO_2 and 95% air. When BMECs were 90% confluent, they were used in the following experiments.

The study was approved by the Animal Ethics Committee of the Nanjing University of Chinese Medicine and the experiment was strictly performed according to the guidelines of laboratory animal care (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council)[19].

Identification of primary BMECs: Typical cobblestone morphology is the feature of BMECs under a microscope and the expression of factor VIII-associated antigen is its specific immunological marker. In the present study, the expression of VIII-associated antigen was detected to identify BMECs[20,21]. BMECs (1×10^5) were seeded in a gelatin-coated 24-well plate with a coverslip on the bottom of each well. The cells were fixed with 4% formaldehyde for 20 min, incubated with 0.1% Triton X-100 for 15 min and 3% H_2O_2 for 30 min successively. After blocked by 10% BSA, BMECs has incubated with primary antibodies of factor VIII-associated antigen (1:1000; Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C and then incubated with HRP-conjugated secondary antibody

(1:500, Wuhan Boster Biological Technology, China) for 30 min at 37°C. After staining by DAB for 3 min, the cells were costained by hematoxylin. The cells with brown cytoplasm were BMECs.

Preparation of OGD/R model *in vitro*: The OGD/R model of BMECs was made as a previous report[22]. The culture media were replaced by a glucose-free DMEM, perfused with 5% CO_2 and 95% N_2 for 2 hrs at 37°C and then normally cultured for 24 hrs to imitate reperfusion injury.

Experiment groups and treatment: In MTT assay, BMECs were randomly divided into 10 groups of control, model, ELC groups (2.0 mg L^{-1}, 10 mg L^{-1}, 50 mg L^{-1} and 250 mg L^{-1}) and BO groups (10 μg L^{-1}, 50 μg L^{-1}, 250 μg L^{-1} and 1.0 mg L^{-1}). Except for the control group, each group was exposed to OGD 2 h/R 24 hrs. Drug treatment groups were treated with corresponding drugs for 3 hrs before OGD/R and during the OGD/R period. The model and control group were administrated by DMSO with equal volume. The OD value was measured at 490 nm via a Synergy-HT microplate reader (Bio-Tek Instruments, USA). The cell viability rate was calculated according to the following formula:

$$\text{Cell viability rate (\%)} = \frac{OD_{experiment} - OD_{blank}}{OD_{control\ group} - OD_{blank}} \times 100\%$$

In the other measurements, BMECs were randomly divided into 5 groups of control, model, ELC (50 mg L^{-1}), BO (50 μg L^{-1}) and ELC+BO (50 mg L^{-1} of ELC with 50 μg L^{-1} of BO) according to the result of MTT assay. The OGD/R injury and treatments were the same as those in MTT assay.

Dose optimization of TMPP and BO by MTT assay: Anti-oxidation ability assays: The levels of SOD, MDA, CAT and GSH-Px in the culture medium were measured via the colorimetric method according to the instructions of the kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The level of intracellular ROS was measured depending on the conversion of non-fluorescent DCFH-DA to its fluorescent derivative. After BMECs were washed with cold PBS, their fluorescence intensities were measured at 535 nm with Synergy-HT microplate reader according to the instructions of the kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Laser confocal Ca^{2+} imaging: After washed with PBS, BMECs were incubated with 5.0 μM of Fluo-3/AM (Biotium, Hayward, CA, USA) for 30 min and washed again with PBS. The fluorescence intensities of BMECs were measured by a laser scanning confocal microscope (Leica TCS-SP5, Solms, Germany). The excitation wavelength was 488 nm and the emission wavelength was from 505-530 nm.

Flow cytometry for apoptosis assay: BMECs were transferred to a reaction tube with a density of 1×10^5 mL^{-1} and incubated with Annexin-V-FITC/PI staining reagent (Beijing Solarbio Science and Technology Co., Ltd, Beijing, China). The percentage of apoptosis (%) was measured using a flow cytometer (Cytomics FC 500MPL, Beckman Coulter, USA). And the measurement was performed according to the manufacturer's instructions to differentiate early apoptosis and late apoptosis.

Real-time PCR assay: After the total RNA of cells was extracted using a Trizol reagent according to the manufacturer's instructions, it was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Then cDNAs for Bcl-2, Bax, p53, caspase-3 and GAPDH were amplified by real-time PCR using the SYBR Premix Ex Taq™ kit (TakaRa Bio Inc., Shiga, Japan) via an MJ Mini thermal cycler (Bio-Rad). The amplification procedure and the primers was the same as our previous study[17].

Western blot for angiogenesis assay: BMECs were harvested and lysed by a cold RIPA Lysis Buffer (Thermo Scientific, Inc. USA) on ice. The lysate was centrifuged at 12,000 rpm for 15 min at 4°C. Part of the supernatant was used for the determination of protein concentration by a BCA protein assay kit (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China), the others were diluted to 7 μg μL^{-1} with loading buffer and heated for 5 min at 95°C. Then, 10 μL (70 μg) of the protein sample was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 2 hrs and then was probed with the following primary antibodies overnight at 4°C: anti-bFGF (1:1000; Santa Cruz Biotechnologies, Dallas, TX, USA), anti-FGFR1 and anti-VEGF (Proteintech Group, Inc, Rosemont, USA), anti-VEGFR1 and VEGFR2 (1:1000; Abcam, Cambridge, UK). Anti-β-actin (1:5000; Bioworld Technology, Inc., St. Louis Park, MN, USA) was used for normalization. After washed with TBST, the membrane was incubated with secondary antibody (goat anti-rabbit or rabbit anti-mouse 1:10,000, Boster Biotechnology, Ltd., Wuhan, China) for 2 hrs. The protein blots were captured by enhanced chemiluminescence method and analyzed using Image-J software.

Statistical analysis: All data were expressed as Mean±standard deviation (SD) and analyzed by SPSS 13.0 software. Statistical differences among multiple groups were determined by one-way Analysis of Variance (ANOVA) followed by Tukey's test for multiple comparisons. A probability of $p<0.05$ was considered significant for all statistical analyses.

RESULTS

Identification of BMECs: As shown in Fig. 1a, the morphology of BMECs under a microscope was a typical cobblestone-like appearance, which was regarded as the feature of BMECs. Brown cytoplasm represented a positive expression of factor VIII-associated antigen, which was the immunological characteristic of BMECs. The immunohistochemical image was shown in Fig. 1b. The purity of BMECs was over 90%.

Dose optimizations of ELC and BO via cell viability assay: Figure 2 showed the results of the dose optimization of ELC and BO. Clearly, OGD/R injured induced a remarkable reduction in cell viability in comparison to the control group ($p<0.01$). Compared to the model group, the treatment of ECL displayed concentration-dependent protection from

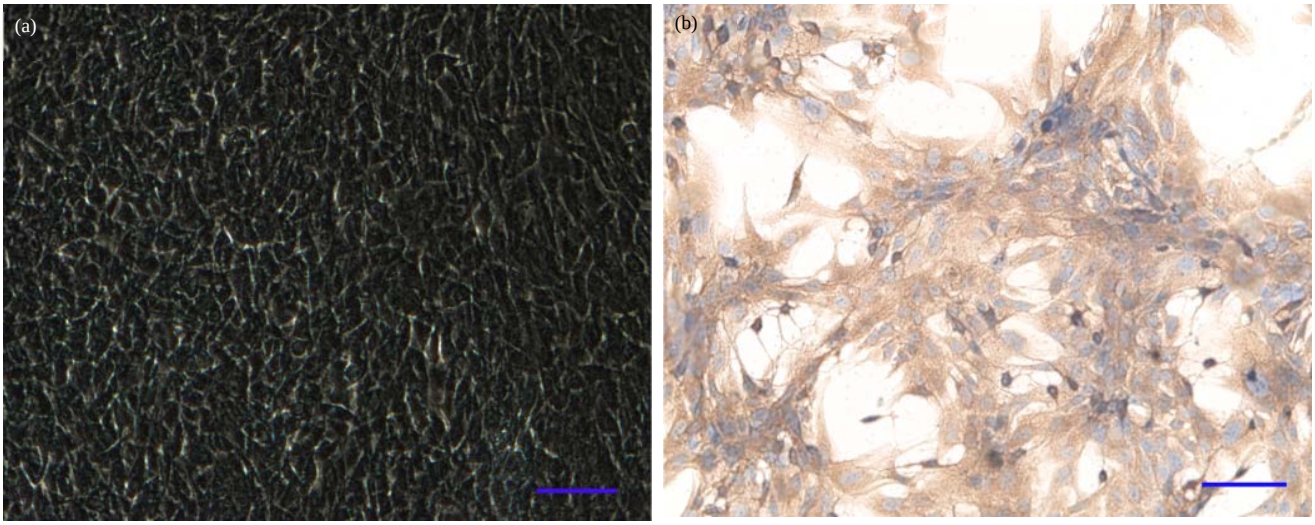

Fig. 1(a-b): The identification of BMECs.
(a) was the representative photograph of BMECs under a microscope. The cells had a typical morphological feature of cobblestone-like appearance, (b) was the representative immuno-cytochemistrical photograph of BMECs. Brown cytoplasm represented a positive expression of factor VIII-associated antigen, an immunological characteristic of BMECs. Scale bar represented 50 μm

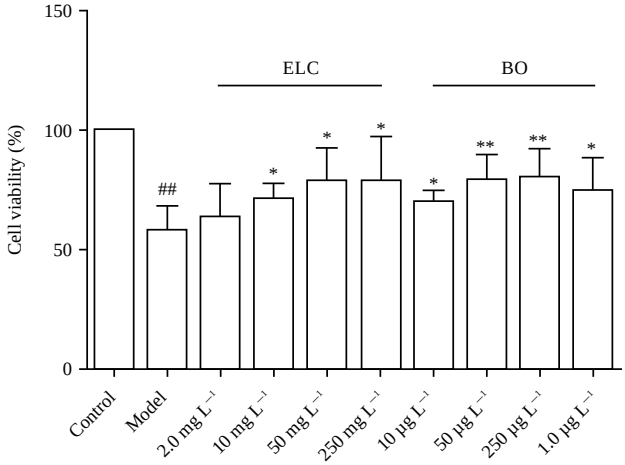

Fig. 2: Dose optimizations of ELC and BO by cell viability assay (n = 6)
Cell viability was evaluated by MTT assay, BMECs with OGD/R injury were treated by indicated concentrations of ELC or BO as shown, Compared to control group, ##p<0.01; compared to model group, *p<0.05, **p<0.01

2.0-50 mg L^{-1} ($p<0.05$). However, 250 mg L^{-1} of ELC did not show a better improvement than 50 mg L^{-1}. BO also exhibited concentration-dependent protection from 10-50 μg L^{-1} ($p<0.05$). Similarly, excessive concentration of BO was unable to display a better effect and even decreased the cell viability reversely, such as 250 μg L^{-1} and 1.0 mg L^{-1} of BO. Obviously, the best treating concentrations of ELC and BO were 50 mg L^{-1} and 50 μg L^{-1}, respectively, which were chosen to be used in the following experiments.

The synergy between ELC and BO on anti-oxidative stress: As shown in Fig. 3a-e, OGD/R injury-induced marked reduction of SOD, CAT and GSH-Px, along with the increase of MDA and ROS (compared to control group, $p<0.01$), which indicated that the formation of oxidative injury. ELC displayed an ideal anti-oxidative activity, including the increase of SOD, CAT and GSH-Px and the decrease of MDA and ROS (Fig. 3a-e), while BO only enhanced the level of CAT (Fig. 3c) (compared to model group, $p<0.05$, 0.01). In comparison to BO group, the

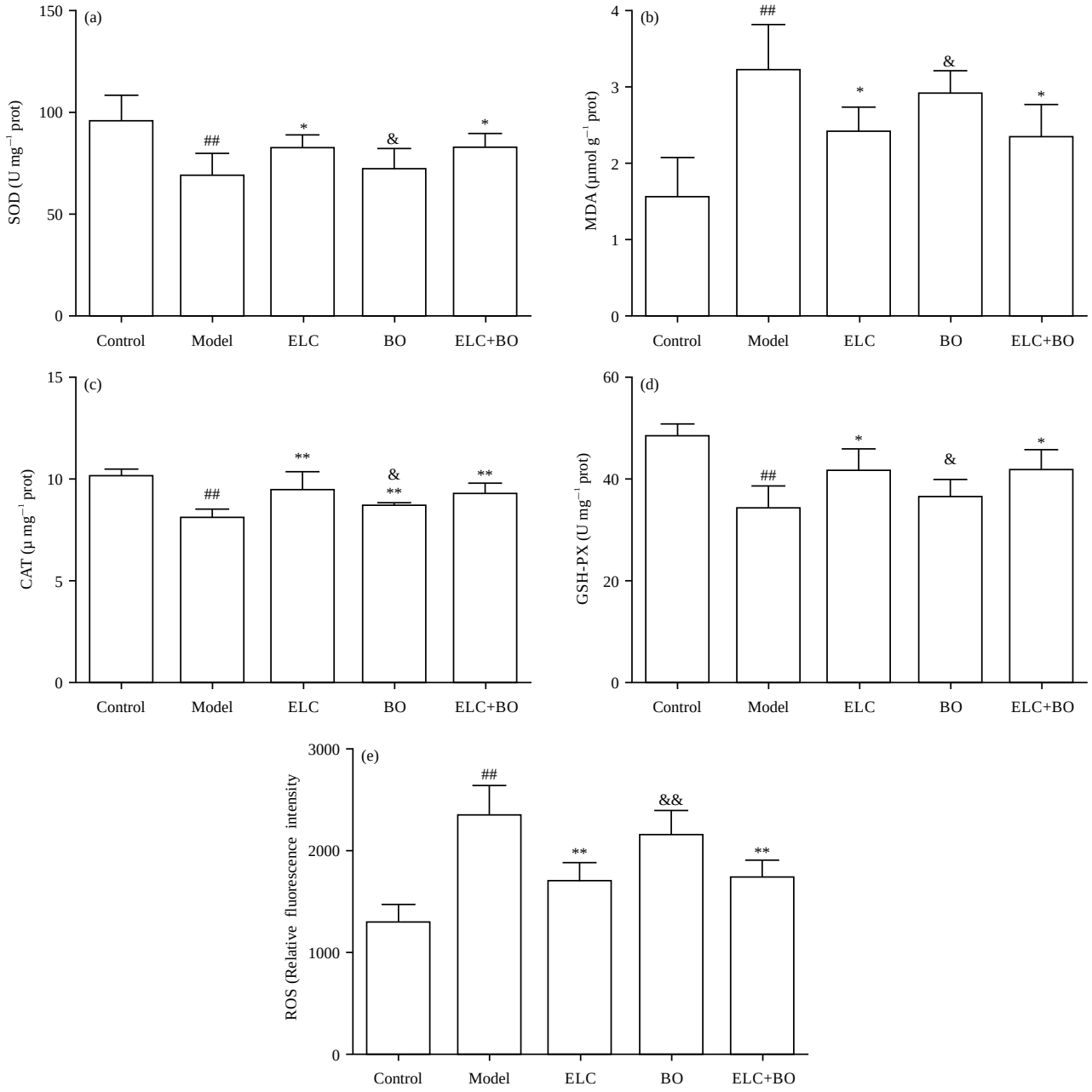

Fig. 3a-e: Synergy between ELC and BO on anti-oxidative stress (n = 6)
(a) SOD, (b) MDA, (c) CAT, (d) GSH-Px and (e) ROS, $^{\#\#}p<0.01$; compared to model group, $^{*}p<0.05$, $^{**}p<0.01$; compared to ELC+BO group, $^{\&}p<0.05$, $^{\&\&}p<0.01$

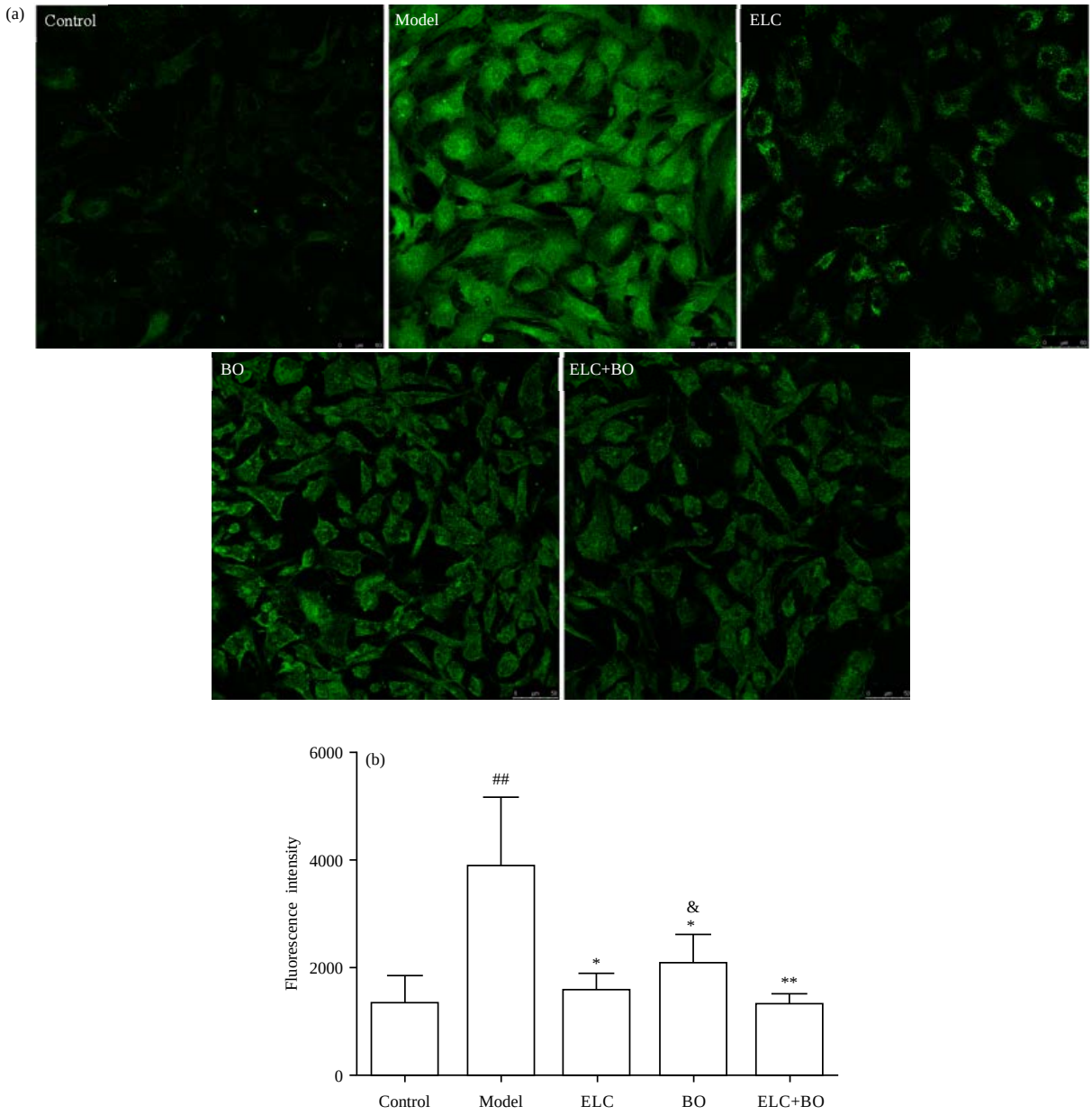

Fig. 4a-b: Synergy between ELC and BO on attenuating Ca^{2+} overload

(a) were representative laser confocal images, (b) were column charts of $[Ca^{2+}]i$ in BMECs of each group (n = 4), $^{\#\#}p<0.01$; compared to model group, *p<0.05, **p<0.01; compared to ELC+BO group, $^{\&}p<0.05$

combination of the ELC and BO showed obvious improvements on SOD, MDA, CAT, GSH-Px and ROS (p<0.05, 0.01), while did not produce a better effect than ELC monotherapy (Fig. 3a-e) (p>0.05).

The synergy between ELC and BO on attenuating Ca^{2+} overload: Ca^{2+} overload is an important feature in BMECs attacked by oxidative stress. The fluorescence intensities denoted $[Ca^{2+}]i$ in Fig. 4a which displayed the synergy between ELC and BO in reducing Ca^{2+} overload. Compared to the control group, OGD/R injury sharply increased the contents of Ca^{2+} in BMECs (p<0.01), which suggested the formation of Ca^{2+} overload. Both ELC and BO had the ability to decrease $[Ca^{2+}]i$ in comparison to the model group (p<0.05). Additionally, their combination further reduced Ca^{2+} overload (p<0.01) and even showed a better effect than BO group (p<0.05), which suggested the synergy between ELC and BO on ameliorating Ca^{2+} overload (Fig. 4b).

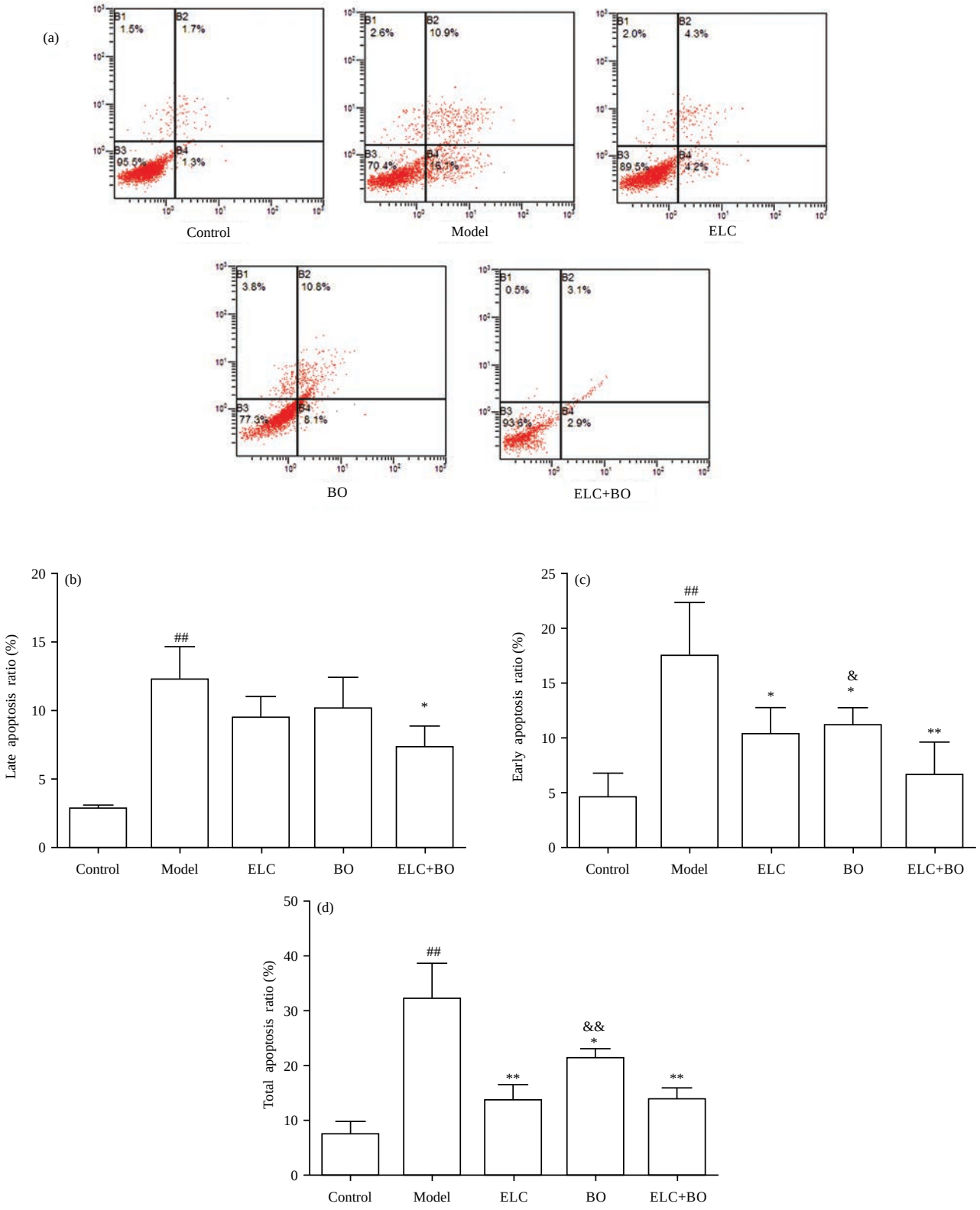

Fig. 5a-d: Synergy between ELC and BO on apoptosis ratio

(a) were representative images of the flow cytometry assay, (b-d) were column charts of the apoptosis percentage of BMECs (n = 4), $^{\#\#}p<0.01$; compared to model group, *p<0.05, **p<0.01; compared to ELC+BO group, $^{\&}p<0.05$

The synergy between ELC and BO on apoptosis ratio: Apoptosis ratio was obtained using a flow cytometer technology and expressed via early apoptosis and late apoptosis (Fig. 5a). As illustrated in Fig. 5b, ODG/R injury caused a sharp increase of early apoptosis, late apoptosis and total apoptosis ($p<0.01$), which confirmed the relationship between apoptosis and ischemic damage. Both ELC and BO reduced early and total apoptosis ratios (Fig. 5c-d) ($p<0.05$, 0.01). Their combining therapy exhibited a more marked apoptosis inhibition than BO monotherapy on early and total apoptosis (Fig. 5c-d) ($p<0.05$, 0.01) and even reduced late apoptosis (Fig. 5b) ($p<0.05$), which indicated their synergy on apoptosis inhibition.

Synergic regulation between ELC and BO on apoptosis-related genes: Figure 6a-d illustrated the synergy of the ELC and BO in regulating apoptosis-related genes, including Bcl-2, Bax, p53 and caspase-3 mRNA. Compared to the control group, OGD/R damage induced a decrease of Bcl-2 mRNA and increases of Bax, p53 and caspase-3 mRNA (Fig. 6a-d) ($p<0.05$), which explained the apoptosis mechanism of OGD/R damage. Treatment of ELC and BO increased Bcl-2 level and reduced Bax (Fig. 6a-b) ($p<0.05$). Moreover, their combination not only displayed a better effect on Bcl-2, Bax and p53 than BO group (Fig. 6a-c), but also even decreased p53 and caspase-3 level in comparison to model group (Fig. 6c-d) ($p<0.05$).

Synergic regulation between ELC and BO on the expressions of angiogenesis-related proteins: Figure 7a illustrated the synergic effect of ELC and BO on the expressions of angiogenesis-related proteins, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), fibroblast growth factor receptor (FGFR), VEGFR1 and VEGFR2. Interestingly, both ELC and BO treatments seemed to further enhance angiogenesis via modulating the expressions of related proteins, including the increases of bFGF, VEGF, FGFR and VEGFR2 and decrease of VEGFR1 by BO (Fig. 7b-f) ($p<0.05$, 0.01) and increase of bFGF by ELC (Fig. 7b) ($p<0.05$).

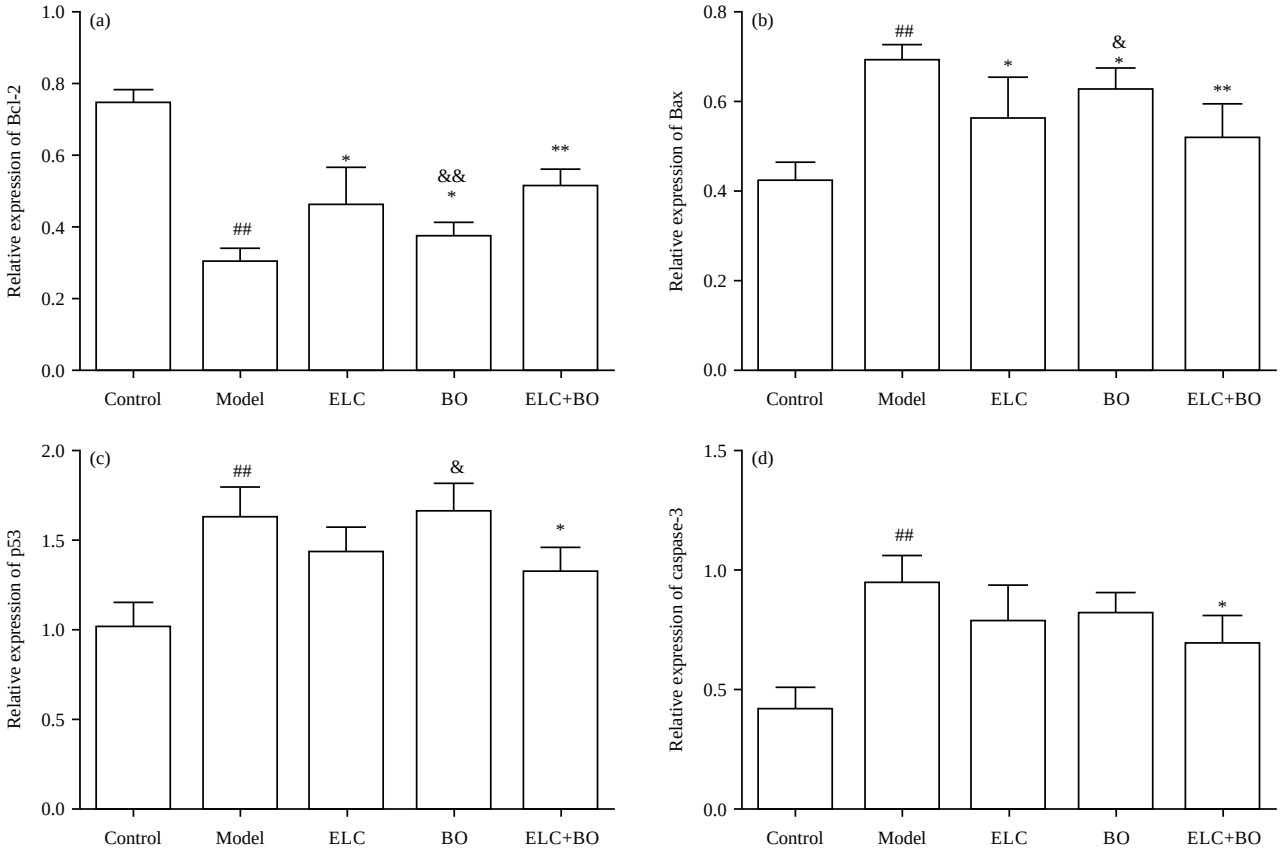

Fig. 6a-d: Synergic regulation between ELC and BO on apoptosis related genes of Bcl-2

(a) Bcl-2, (b) Bax, (c) p53 and (d) caspase-3 mRNA (n = 4), Compared to control group, $^{\#\#}p<0.01$; compared to model group, $^{*}p<0.05$, $^{**}p<0.01$; compared to ELC+BO group, $^{\&}p<0.05$, $^{\&\&}p<0.01$

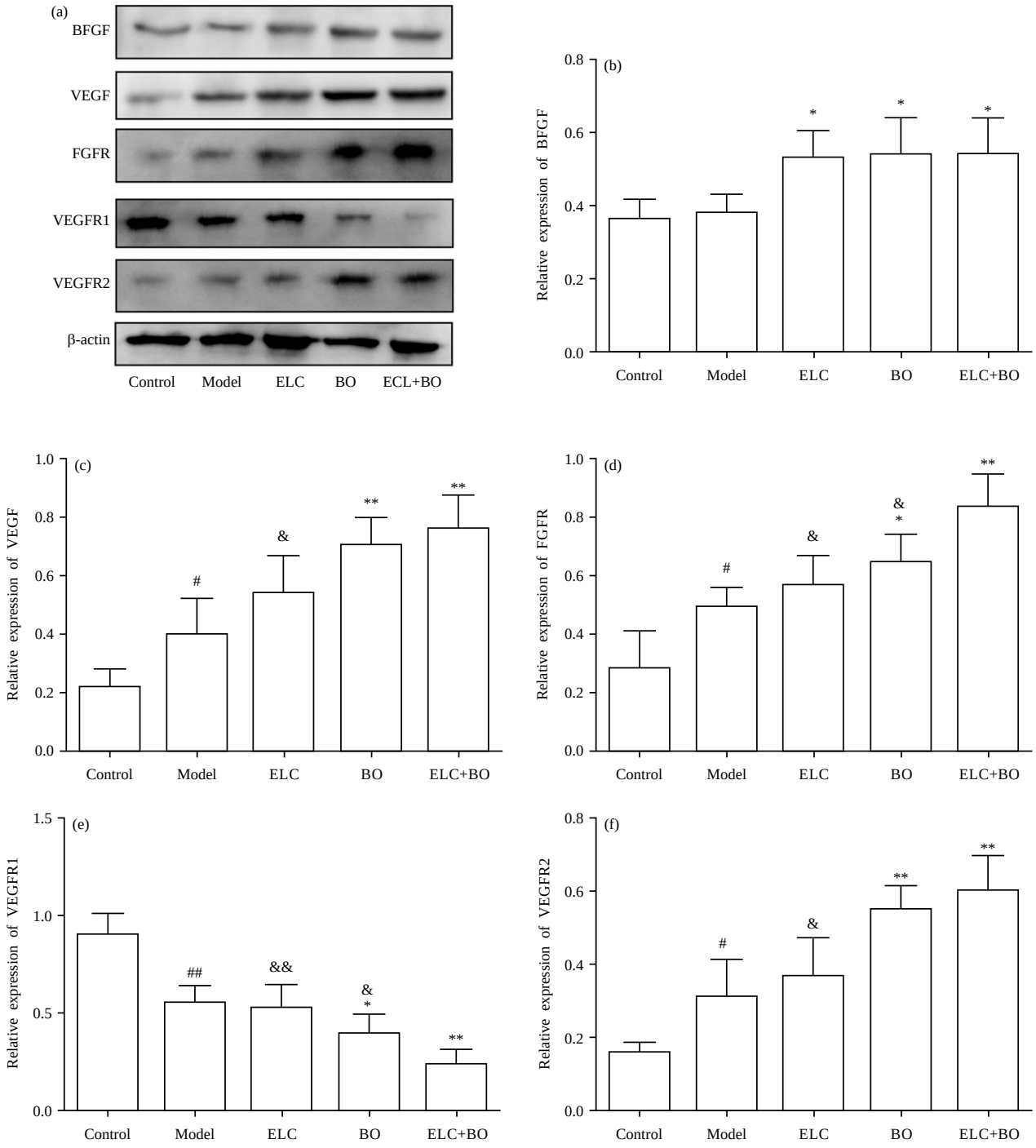

Fig. 7a-f: Synergic regulation between ELC and BO on the expressions of angiogenesis related proteins

(a) Representative western blot images of each protein. (b) Column charts of the expressions of the proteins in each group, (b) bFGF, (c) VEGF, (d) FGFR, (e) VEGFR1 and (f)VEGFR2) (n = 4), Compared to control group, $^{\#}p<0.05$; compared to model group, $^{*}p<0.05$, $^{**}p<0.01$; compared to ELC+BO group, $^{\&}p<0.05$, $^{\&\&}p<0.01$

In comparison to the control group, the model group increased the expressions of VEGF, FGFR and VEGFR2 (Fig. 7c-d and f) ($p<0.05$) and decreased VEGFR1 (Fig. 7e) ($p<0.01$), which indicated that OGD/R injury-induced angiogenesis. Similarly, their combination also displayed a marked synergic effect which was illustrated by further increased VEGF, FGFR

and VEGFR2 (Fig. 7c-d and f) and further decreased VEGFR1 (Fig. 7e) in comparison to ELC monotherapy ($p<0.05$, 0.01). Additionally, their combination further increased FGFR and decreased VEGFR1 in comparison to BO monotherapy (Fig. 7d-e) ($p<0.05$).

DISCUSSION

The synergic therapy of ELC and BO against OGD/R damage on BMECs was evaluated in the present study. It was found that ELC was good at inhibiting oxidative stress and apoptosis, regulating $[Ca^{2+}]i$, while BO preferred to promote angiogenesis. The combination of the two medications produced better therapy than their monotherapies.

The brain is one of the most sensitive organs to hypoxia. If thrombolytic treatment is out of the ideal therapy window, cerebral ischemia/reperfusion arises. BMECs OGD/R injury simulates the clinic ischemic stroke and is widely used for exploring the mechanism of cerebral ischemic disease[4]. Normally, mitochondrial electron transport chains produced a small amount of ROS, which are necessary for cellular well-being and will be eliminated by some endogenous antioxidant enzymes, such as SOD, CAT and GSH-Px. However, excess ROS can damage nucleic acids, proteins, cellular membrane and even trigger a variety of pathological reactions[23,24]. The present experiment showed that ELC had an ideal anti-oxidative activity on OGD/R injured BMECs. Specifically, ELC increased SOD, CAT and GSH-Px and decreased MDA and ROS and the major active constituents might be TMP and ligustilide in ELC according to the previous reports[7,9]. However, BO only enhanced the level of CAT. Moreover, their combination produced a better therapy than BO, which indicated their synergic effect.

As a second messenger, Ca^{2+} involves many extracellular signaling cascades and plays a critical role in cell survival and function. However, excess accumulation of intracellular Ca^{2+}, known as Ca^{2+} overload, will reversely induce a series of reverse actions, such as activation of apoptosis, the formation of oxidative stress and excitotoxicity[25,26]. Its oxidative injury on cell membrane can enhance cellular permeability and further increase the influx of Ca^{2+}. Specifically, it was reported that ROS also mediates the activity of Ca^{2+} channels located at the plasma membrane, the mitochondria and the reticulum by a cross-talk manner[27]. The present study suggested that both of the monotherapies of ELC and BO decreased $[Ca^{2+}]i$ in BMECs damaged by OGD/R and the result confirmed their bioactivity of alleviating Ca^{2+} overload. Additionally, their combination made a more marked reduction of $[Ca^{2+}]i$ than BO group. The present research also verified Lei's report which demonstrated that ligustrazine might act as a blocker of voltage-operated Ca^{2+} channels (VDCCs) and senkyunolide A act as an inhibitor of ryanodine receptors (RYRs) and VDCCs[28].

Apoptosis, a common manner of cell death, displays a multi-stage process, including early-stage and late stage. Furthermore, it had been proved that both Ca^{2+} overload and hypoxia may induce cellular apoptosis cascade reaction via modulating the transcription of apoptosis-related genes[29]. Bcl-2 family is regarded as one of the most important factors regulating apoptosis, while the ratio of Bax to Bcl-2 acts as a switch to determine cell susceptibility to apoptosis[30]. Additionally, caspase-dependent apoptosis also plays a key role in cell death, especially caspase-3 which is considered to be a decisive factor in the apoptotic process. So, the expression of caspase-3 is frequently used to be a marker to evaluate apoptosis status[31]. The gene of p53 stimulates a wide network of signals through an extrinsic and intrinsic apoptotic pathway. The extrinsic pathway triggers the activation of the caspase cascade. The intrinsic mitochondrial pathway shifts the balance in the Bcl-2 family towards the pro-apoptotic members and promotes the formation of apoptosomes and caspase-mediated apoptosis successively. The present study demonstrated that ELC and BO reduced early and total apoptosis ratios. The monotherapies of ELC and BO only increased the transcription of Bcl-2 and reduced that of Bax. Yet, their combination even reduced p53 and caspase-3 levels. Apparently, their synergy on anti-apoptosis was exhibited well via modulating the transcriptions of apoptosis-related genes. The present studies had confirmed the active ingredients in ELC inhibiting apoptosis might be liguzinediol and senkyunolide I[32,33].

Angiogenesis is a multi-step process beginning with the degradation of the basement membrane of activated endothelial cells and these cells will migrate, proliferate and form solid endothelial cell sprouts into the stromal space. Although the adult brain is completely developed and stable, some pathological conditions, such as hypoxia, ischemia and brain tumor growth, may trigger angiogenesis acting as a compensatory mechanism[34]. It has been proved that a series of growth factors are involved in angiogenesis, including VEGF, bFGF and their relevant receptors. As one of the most important stimulators of angiogenesis, VEGF regulates endothelial cell proliferation, migration and tube formation by activating its receptors of VEGFR1 and VEGFR2[35,36]. Commonly, VEGFR2 is considered to be more effective to VEGF-induced

angiogenesis[37]. Activation of VEGFR-2 induces the phosphorylation of many downstream proteins, amplification of angiogenesis-stimulating cascades and finally the formation of neovascular. Thus, VEGFR-2 has been explored as an important therapeutic target for the development of ischemia protection and anticancer agents[38]. VEGFR1 is mainly expressed in monocytes, macrophages and hematopoietic stem cells. Although the affinity of VEGFR1 to VEGF is ten times higher than VEGFR2, VEGFR1 has much less tyrosine kinase activity. So VEGFR1 may act as a reverse regulator of VEGFR2[39]. FGFs exert their bioactivities by binding to FGFR to activate tyrosine kinase. Previous studies showed that FGF/FGFR is critical for construct endothelial networks and provides a pattern for the vasa vasorum to form a plexus-like network via a series of pro-angiogenic reactions[40]. In this study, we found that OGD/R injury increased the expressions of VEGF, FGFR and VEGFR2 and decreased that of VEGFR1, which indicated the appearance of angiogenesis as the previous reports[34]. Interestingly, treatments of BO further enhanced angiogenesis via increases of bFGF, VEGF, FGFR and VEGFR2 and decrease of VEGFR1. However, ELC only enhanced bFGF level. Moreover, their combination displayed a marked synergic effect. Specifically, the combined therapy further modulated VEGF, FGFR, VEGFR1 and VEGFR2 levels in comparison to ELC monotherapy and FGFR and VEGFR1 in comparison to BO monotherapy. Additionally, a recent report also demonstrated that both ligustrazine in ELC and BO might be the effective compounds inducing angiogenesis[41,42].

The present study not only discovered that the protective mechanism of ELC and BO combination was involved in anti-oxidation, apoptosis inhibition, Ca^{2+} overload attenuation and angiogenesis but also indicated that ELC was good at attenuating the damage of BMECs, while BO preferred angiogenesis. It is well known that neurogenesis also play a crucial role in improving brain function suffered from an ischemic attack. It is not clear whether the combination is workable on neurogenesis and it will be explored in our future research.

CONCLUSION

The synergic protective effect between ELC and BO against BMECs OGD/R injury was evaluated in this study. It was found that ELC not only had the obvious advantage on anti-oxidative stress, but also had a certain degree of superiority in decreasing apoptosis via modulation of apoptosis-related genes levels and alleviating Ca^{2+} overload. BO seemed to have superiority on angiogenesis. Moreover, their combining therapy brought a new target of VEGFR1 on angiogenesis which was excluded in their monotherapies. The results provided a scientific basis for the combined application of ELC and BO in ischemic stroke treatment.

SIGNIFICANCE STATEMENT

The study explored the synergic mechanism between ELC and BO against oxygen-glucose deprivation/reperfusion (OGD/R) injury. ELC and BO had their respective superiorities in their treatment. The study provides future researchers with a new perspective to explore the synergic effect between the treatments for cerebral ischemia.

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REFERENCES

1. Katan, M. and A. Luft, 2018. Global burden of stroke. Semin. Neurol., 38: 208-211.
2. Beck H. and K. H. Plate 2009. Angiogenesis after cerebral ischemia. Acta Neuropathol., 117: 481-496.
3. Chen J. and M. Chopp, 2007. Neurorestorative treatment of stroke: Cell and pharmacological approaches. NeuroRX., 3: 466-473.
4. Huang S., T. Gong, T. Zhang, X. Wang, Q. Cheng and Y. Li, 2018. Zhongfenggao Protects Brain Microvascular Endothelial Cells from Oxygen-Glucose Dep.rivation/Reoxygenation-Induced Injury by Angiogenesis. Biol. Pharm. Bull., 42: 222-230.
5. Yang, X., X. Zeng and T. Wu, 2016. Chuanxiong preparations for preventing stroke. Cochrane Database Syst. Rev. 10.1002/14651858.CD006765.pub2
6. Ran, X., L. Ma, C. Peng, H. Zhang and L.P. Qin, 2011. *Ligusticum chuanxiong* Hort: A review of chemistry and pharmacology. Pharm. Biol., 49: 1180-1189.
7. Chen, Z., C. Zhang, F. Gao, Q. Fu, C. Fu, Y. He and J. Zhang, 2018. A systematic review on the rhizome of *Ligusticum chuanxiong* Hort. (Chuanxiong). Food Chem. Toxicol., 119: 309-325.

8. Chang, L., C.Y. Yin, H.Y. Wu, B.B. Tian, Y. Zhu, C.X. Luo and Dong-YaZhu, 2017. (+)-Borneol is neuroprotective against permanent cerebral ischemia in rats by suppressing production of proinflammatory cytokines. J. Biomed. Res., 31: 306-314.
9. Zhang, X.G., C. Shan, J.Z. Zhu, X.Y. Bao and Q. Tong *et al*., 2018. Additive neuroprotective effect of borneol with mesenchymal stem cells on ischemic stroke in mice. Front. Physiol., 10.3389/fphys.2017.01133
10. Yu, B., M. Ruan, X.B. Cui, J.M. Guo, L. Xu and X.P. Dong, 2013. Effects of borneol on the pharmacokinetics of geniposide in cortex, hippocampus, hypothalamus and striatum of conscious rat by simultaneous brain microdialysis coupled with UPLC-MS. J. Pharm. Biomed. Anal., 77: 128-132.
11. Yu, B., M. Ruan, Y. Sun, X. Cui, Y. Yu, L. Wang and T. Fang, 2011. Effect of borneol and electroacupuncture on the distribution of hyperforin in the rat brain. Neural Regen. Res., 6: 1876-1882.
12. Yu, B., M. Ruan, X. Dong, Y. Yu and H. Cheng, 2013. The mechanism of the opening of the blood-brain barrier by borneol: A pharmacodynamics and pharmacokinetics combination study. J. Ethnopharmacol., 150: 1096-1108.
13. Kong, Q.X., Z.Y. Wu, X. Chu, R.Q. Liang, M. Xia and L. Li, 2013. Study on the anti-cerebral ischemia effect of borneol and its mechanism. Afr. J. Tradit. Complement. Altern. Med., 11: 161-164.
14. Wu, H.Y., Y. Tang, L.Y. Gao, W.X. Sun and Y. Hua *et al*., 2014. The synergetic effect of edaravone and borneol in the rat model of ischemic stroke. Eur. J. Pharmacol., 740: 522-531.
15. Liu, R., L. Zhang, X. Lan, L. Li, T.T. Zhang, J.H. Sun and G.H. Du, 2011. Protection by borneol on cortical neurons against oxygen-glucose deprivation/reperfusion: Involvement of anti-oxidation and anti-inflammation through nuclear transcription factor κappaB signaling pathway. Neuroscience, 176: 408-419.
16. Dong, T., N. Chen, X. Ma, J. Wang, J. Wen, Q. Xie and R. Ma, 2018. The protective roles of L-borneolum, D-borneolum and synthetic borneol in cerebral ischaemia via modulation of the neurovascular unit. Biomed. Pharmacother., 102: 874-883.
17. Yu B., M. Ruan, T. Liang, Y. Yu, 2020. Synergy Between Borneol and Extract of *Ligusticum chuanxiong* Hort Against Cortex and Striatum Ischemia. International J. of Pharmacology. 16: 104-119.
18. Liu Y., Q. Xue, Q. Tang, M. Hou, H. Qi, G. Chen, W. Chen, J. Zhang, Y. Chen and X. Xu, 2013. A simple method for isolating and culturing the rat brain microvascular endothelial cells. Microvascular Research. 90: 199-205.
19. Bocci G., 2002. In-vitro evidence of autocrine secretion of vascular endothelial growth factor by endothelial cells from human placental blood vessels. Molecular Human Reproduction, 7: 771-777.
20. Lathey J. L., C. A. Wiley, M. A. Verity and J. A. Nelson, 2004. Cultured human brain capillary endothelial cells are permissive for infection by human cytomegalovirus. Virology. 176: 266-273.
21. Yang H., X. Xi, B. Zhao, Z. Su and Z. Wang, 2017. KLF4 protects brain microvascular endothelial cells from ischemic stroke induced apoptosis by transcriptionally activating MALAT1. Biochemical and Biophysical Research Communications. 495: 2376-2382.
22. Hamanaka, R.B. and N.S. Chandel, 2010. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem. Sci., 35: 505-513.
23. Sena, L.A. and N.S. Chandel, 2012. Physiological roles of mitochondrial reactive oxygen species. Mol. Cell, 48: 158-167.
24. Peng T. I. and M. J. Jou, 2010. Oxidative stress caused by mitochondrial calcium overload. ANNALS of the New York academy of Sciences. 1201: 183-188.
25. Szydlowska, K. and M. Tymianski, 2010. Calcium, ischemia and excitotoxicity. Cell Calcium, 47: 122-129.
26. Yang J., 2018. The role of reactive oxygen species in angiogenesis and preventing tissue injury after brain ischemia. Microvascular Research. 123: 62-67.
27. Lei W., J. Ni, X. Xia, M. Jiang and G. Bai, 2018. Searching for synergistic calcium antagonists and novel therapeutic regimens for coronary heart disease therapy from a Traditional Chinese Medicine, Suxiao Jiuxin Pill. Journal of Chromatography B. 1092: 220-227.
28. Jahani-Asl A., M. Germain and R. S. Slack 2009. Mitochondria: Joining forces to thwart cell death. Biochim. Biophys. Acta, Mol. Basis Dis., 1802: 162-166.
29. Wang, G.H., R. Lan, X.D. Zhen, W. Zhang, J. Xiang and D.F. Cai, 2014. An-Gong-Niu-Huang Wan protects against cerebral ischemia induced apoptosis in rats: up-regulation of Bcl-2 and down-regulation of Bax and caspase-3. J. Ethnopharmacol., 154: 156-162.
30. Zhang, Y., R. Lan, J. Wang, X.Y. Li and D.N. Zhu *et al*., 2015. Acupuncture reduced apoptosis and up-regulated BDNF and GDNF expression in hippocampus following hypoxia-ischemia in neonatal rats. J. Ethnopharmacol., 172: 124-132.
31. Hu Y., M. Duan, S. Liang, Y. Wang and Y. Feng, 2015. Senkyunolide I protects rat brain against focal cerebral ischemia-reperfusion injury by up-regulating p-Erk1/2, Nrf2/HO-1 and inhibiting caspase 3. Brain Res., 1605: 39-48.
32. Li Y., P. Song, Q. Zhu, Q. Y. Yin, J. W. Ji, Wei Li, H. M. Bian, 2014. Liguzinediol improved the heart function and inhibited myocardial cell apoptosis in rats with heart failure. Acta Pharmacol Sin., 35: 1257-1264.
33. Plate K. H., 2008. Mechanisms of Angiogenesis in the Brain. J. Neuropathol. Exp. Neurol., 58: 313-320.

34. CHEN Z., X. H. XU and J. HU 2016. Role of pericytes in angiogenesis: focus on cancer angiogenesis and anti-angiogenic therapy. neo., 63: 173-182.
35. Ferrara, N., H.P. Gerber and J. LeCouter, 2003. The biology of VEGF and its receptors. Natl. Med., 9: 669-676.
36. Zhang, Z. and B. Zhou, 2013. Accelerated coronary angiogenesis by Vegfr1-knockout endocardial cells. PLoS One, Vol. 8. 10.1371/journal.pone.0070570
37. Ferrara, N., 2004. Vascular endothelial growth factor: Basic science and clinical progress. Endocr. Rev., 25: 581-611.
38. Sato Y., 2008. VEGFR1 for Lymphangiogenesis. ATVB., 28: 604-605.
39. Woad K.J., A.J. Hammond, M. Hunter, G.E. Mann, M.G. Hunter and R.S. Robinson, 2009. FGF2 is crucial for the development of bovine luteal endothelial networks in vitro. Reprod., 138: 581-588.
40. S. Liao, L. Han, X. Zheng, X. Wang, P. Zhang, J. Wu, R. Liu, Y. Fu, J. Sun, X. Kang, K. Liu, T. P. Fan, S. Li, X. Zheng, 2019. Tanshinol borneol ester, a novel synthetic small molecule angiogenesis stimulator inspired by botanical formulations for angina pectoris Br J Pharmacol., 176: 3143-3160.
41. Mu Q. C., H. Y. Huang, H. J. Gao, P. F. Liu, P. W. Li, Z. Y. Huang, F. B. Yu, T. Lei, Y. Chen and Y. Cheng, 2015. Ligustrazine monomer against cerebral ischemia-reperfusion injury. Neural Regen. Res., 10: 832-840.