Research Article

Long-Zhi Decoction Medicated Serum Promotes Angiogenesis in Human Umbilical Vein Endothelial Cells Based on Autophagy

Quan He, Qihua Liu, Yongbin Chen, Jiaquan Meng, and Ling Zou

Department of Traditional Chinese Medicine, First Affiliated Hospital of Guangxi Medical University, Nanning, China

Correspondence should be addressed to Yongbin Chen; cyb3506633@126.com

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Ischemic stroke (IS) is a fatal subtype of stroke that lacks effective treatments. Angiogenesis following IS is an effective response that mediates brain recovery and repair. Our previous study demonstrated that long-zhi decoction (LZD), a Chinese herbal formula, promoted angiogenesis in rats of IS model. To further investigate the association between the proangiogenic mechanism of an LZD-medicated serum and cellular autophagy, we evaluated its promotional effect on angiogenesis in human umbilical vein endothelial cells (HUVECs) in vitro. We used HUVECs subjected to \( \text{H}_2\text{O}_2 \) to induce injury and observed the effects of the LZD-medicated serum treatment. Cell-based assays included proliferation, migration, and tube formation. To assess the extent of autophagy, transmission electron microscopy was used to measure the number of autophagosomes. Immunofluorescence and Western blotting were performed to evaluate the autophagy-related protein of LC3-II and Beclin-1. The LZD-medicated serum promoted proliferation, migration, and tube formation in HUVECs. The LZD-medicated serum also increased the autophagosomes and the autophagic protein expressions of LC3-II and Beclin-1. The proangiogenic and autophagic activity of LZD provides new cogitations to its clinical application and may lead to potential drug development for treating various vascular diseases, especially in the elderly, in the future.

1. Introduction

Ischemic stroke (IS) is a transient episode of a neurological dysfunction caused by the loss of blood flow to the brain with acute infarction, and it has a high morbidity and mortality in the world [1–3]. IS can lead to neurological impairment, brain tissue ischemia, hypoxia, and a serious clinical complication [4]. Therapeutic angiogenesis may enhance the supply of oxygen and nutrients to the affected tissue, thus making it beneficial to the recovery of IS, and extensive evidence suggests that it may be a promising antistroke therapeutic strategy [5]. Recent research has shown that a new vessel formation after IS not only replenishes blood flow to the ischemic area of the brain but also improves neurological functions in both clinical trials and animal models [6, 7]. Moreover, angiogenesis and cell autophagy have a high degree of correlation, and the link between them has received much attention in the current literature [8, 9].

Autophagy (or cellular “self-eating”) plays a pivotal role in various aspects of cell physiology, but flaws in this process are associated with numerous pathological conditions [10]. Autophagy is widespread in eukaryotes, is constitutively at a low level, and is a highly evolutionarily conserved process essential for protecting cellular survival, homeostasis, and integrity [11]. However, different stress conditions involving starvation or organelle deterioration changing nutrient conditions, hypoxia, and pathogen infection result in the upregulation of this process [12]. More importantly, abnormal autophagy is associated with human pathologies, such as cardiovascular, diabetes, neurodegeneration, cancer, pulmonary, and macular degeneration. Cell autophagy plays an important role in a variety of clinical diseases, but the exact mechanism remains unclear [13–15]. Therefore, ongoing work on this topic is necessary to further determine the cellular factors regulating this inherently complex process.

Long-zhi decoction (LZD), a clinical Chinese herbal formula, consists of nine herbs: Huangqi (Radix Astragali seu Hedysari), Shuizhi (Whitmania Pigra Whitman), Chi Shao (Radix Paeoniae Rubra), Niuxi (Achyranthes Bidentata), Danggui (Radix Angelica Sinensis), Taoren (Semen persicae),
Dilong (Pheretima), Honghua (Flos Carthami), and Chuanxiong (Rhizaoma Ligustici Chuanxiong). Our research team had already found that LZD could significantly accelerate the rate of capillary formation and promote the recovery of neurological function in an acute ischemic-damaged mouse model compared with the control. However, the mechanisms of this drug for stroke treatment are still unclear. In the present study, we aim to observe the angiogenesis effects and the autophagy changes in LZD on human umbilical vein endothelial cells (HUVECs).

2. Materials and Methods

2.1. Ethics Statement. All processes in the present research were allowed in accordance with the National Institute of Health Guide and Use of Laboratory Animals, which was also approved by the care of Experimental Animals Committee of the Guangxi Medical University (Nanning, China).

2.2. Cell Culture and Chemicals. HUVECs were purchased from the American Type Culture Collection (ATCC®, Manassas, VA, USA). The cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (GIBCO®, Gaithersburg, MD, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin in humidified 5% CO₂ at 37°C. HUVECs at the early passages were used in all the experiments and cultured in gelatin-coated plates with a complete growth medium. For transfection, the cells were grown up to 90% confluence and intervened with H₂O₂ using a concentration of 400 μmol/L (AR, Nanning Wilking Biological Technology Co., Ltd., China) before incubation with DMEM for 4 h to establish the base model for the entire experiment. At the completion of the experiments, the cells were washed with precooling phosphate-buffered saline (PBS) for the subsequent experiments.

2.3. Preparation of the LZD-Medicated Serum. The LZD-medicated serum was manufactured according to an elapsed report [16, 17]. Twenty Sprague-Dawley rats were randomly divided into the LZD group (n = 10) and the control group (n = 10). The rats were housed in a 20°C–25°C air-conditioned room with a 12 h light-dark cycle and provided with a standard diet with free access to tap water. The rats in the LZD group underwent intragastric administration of LZD (7.575 g/kg) two times a day for five days. The rats in the blank serum group received intragastric administration of physiological saline twice a day for five days. The volume of intragastric administration of the two groups was consistent. After 1 h after the final administration, the rats were anesthetized using chloral hydrate intraperitoneally and then sacrificed by an intraperitoneal injection of an overdose of chloral hydrate. The rats’ blood samples (10 ml) were collected from the abdominal aorta and centrifuged at 3000 × g at 4°C for 15 min. The serum was isolated and stored at −80°C until further analysis.

2.4. Cell Proliferation Assay. The cell suspension of HUVECs was inoculated into a 96-well plate at a density of 5 × 10⁵ cells/mL. The HUVECs were divided into four groups: normal control group, blank serum group, H₂O₂ group, and H₂O₂ with LZD-mediated serum group. Following treatment, cell proliferation was evaluated using the Cell Counting Kit-8 (CCK8) reagent (Dojindo Laboratories, Kumamoto, Japan). According to the manufacturer's protocol (Nanning Wilking Biological Technology Co., Ltd., China), the absorbance values were read at 450 nm using a plate reader (Thermo Scientific, Watertown, USA).

2.5. Wound Scratch Test. Wound scratch test was applied to investigate the migration capability of HUVECs after each group of drug intervention. HUVECs were seeded in 24-well plates and fed with low-glucose DMEM containing 10% FBS. After the cells were grown to approximately 100%, the surface-adherent cells were wounded with a pipette tip. Then, the cells that fell off were flushed with PBS to wash away the floating cells and cellular debris and then cultured with low-glucose DMEM containing intervention drugs. The initial wounding and the migration of HUVECs in the scratched area were observed under an inverted microscope for 24 h. Three different fields from each sample were photographed and quantitatively analyzed by counting the cell number between the borderlines.

2.6. In Vitro Network Formation Assay. HUVECs (1 × 10⁵ cells/well) in different drug groups were seeded in a 96-well plate precoated with Matrigel (50 μL/well, BD Biosciences, Bedford, MA 01730, USA) and transferred to a cell incubator with humidified 5% CO₂ at 37°C for 24 h. The network structure was observed and imaged under an inverted microscope at 40x magnification. The angiogenic activities were evaluated by counting the branch points of the network structure formed, and the average numbers of branch points were measured from three random fields.

2.7. Transmission Electron Microscopy (TEM). In observing the autophagosomes in HUVECs, HUVECs were harvested and fixed with 2.5% glutaraldehyde at 4°C for 2 h. The samples were then suspended in PBS containing 1% osmic acid at 4°C for 2 h. Following dehydration and embedding [18], ultrathin sections (60–70 nm) were prepared on uncoated copper grids using an Ultrotome and stained with uranyl acetate and lead citrate. Images were photographed using a TEM (H7650, Hitachi Limited, Japan).

2.8. Immunofluorescence Microscopy. Immunofluorescence was applied to assess the expression of autophagy protein, such as microtubule-associated protein 1A/1B-LC3-II, in the HUVEC samples from the normal, blank serum, H₂O₂, and H₂O₂ with LZD serum groups. Cells (2 × 10⁴) were seeded on 14 mm plates and cultivated for 24 h prior to intervention. Following three washes with PBS, the cells were then fixed with 4% paraformaldehyde at room temperature for half an hour and then washed three times with PBS. After permeabilization with 1% BSA/0.05% Triton X-100, the cells of the four groups were incubated with antibodies against LC3 (1:400, Cell Signaling Technology, USA) and a humified container at 4°C for 12 h. After washing three
Figure 1: Inhibitory effect of H$_2$O$_2$ on HUVECs proliferation. HUVECs, human umbilical vein endothelial cells; LZDMS, long-zhi decoction medicated serum. (a) Proliferation inhibition of HUVECs with different concentrations of H$_2$O$_2$ for 4 h. (b) Proliferation inhibition of HUVECs with 400 µmol·L$^{-1}$ of H$_2$O$_2$ at a series of time points. (c) The effect of each experimental group on HUVECs proliferation. Values are expressed as the mean ± standard error of the mean (n = 3). **P < 0.01 and *P > 0.05, compared with the control group; ##P < 0.01, compared with the control group and blank serum group; △P < 0.01, compared with the H$_2$O$_2$ group. LZDMS, long-zhi decoction medicated serum.

2.9. Western Blot Analysis. HUVECs were cultured in 6-well plates with $1 \times 10^6$ cells per plate. The cells were treated with different drug groups for 24 h and lysed with 80 µl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 1% SDS, and 5% 2-mercapto-ethanol). The protein samples collected above were boiled at 100°C for 10 min before loading onto the acrylamide gel (12%) for SDS-polyacrylamide gel electrophoresis. A biotinylated ladder (Cell Signaling Technology, Danvers, MA, USA) was used as a size marker. After the proteins were separated by SDS-PAGE (Beyotime Biological Co., Ltd., China), they were transferred onto a nitrocellulose membrane at 90 V for 100 min. The membrane was blocked with a commercial protein blocking solution for 1 h after the blotting procedure, followed by washing three times with 1x Tris-buffered saline (TBS, 20 mM Tris-HCl, 136 mM NaCl, pH 7.6) containing 1% Tween-20 times with PBS, the sections were incubated with Alexa Fluor® 488-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) secondary antibody (1:1,000, Cell Signaling Technology, USA) for 1 h at room temperature and then incubated with 0.0001% 4,6-diamidino-2-phenylindole (Sigma-Aldrich) for 10 min. After washing, the plates were examined under a fluorescence microscope [19].
for 10 min. Then, the membrane was incubated overnight at 4°C with anti-LC3 (1:1,000, Cell Signaling Technology, USA), anti-Beclin-1 (1:1,000, Abcam, UK), and Anti-β-Actin (1:2,000, Santa Cruz Biotechnology, Inc., USA). On the next day, the membrane was washed three times with TBS for 15 min, followed by incubation with Goat Anti-Rabbit IgG (H + L) antibody (1:15,000, Cell Signaling Technology, USA) for 1 h at room temperature. After the membrane was washed three times with TBS for 15 min, the electrochemiluminescence detection system was used to analyze the blots.

### 3. Results

3.1. \( \mathrm{H}_2 \mathrm{O}_2 \) Inhibits Cell Proliferation and LZD-Medicated Serum Significantly Promotes Injured HUVEC Proliferation. CCK8 assay was used to investigate cell proliferation. The optimal dosage and intervention time of \( \mathrm{H}_2 \mathrm{O}_2 \) were determined by a preexperiment. The condition in which 400 \( \mu \mathrm{mol} / \mathrm{L} \) of \( \mathrm{H}_2 \mathrm{O}_2 \) was used to interfere with HUVECs for 4 h was suitable for the experiment (Figures 1(a)-1(b)) to determine whether LZD-medicated serum in HUVECs affected cell proliferation. The results showed that the cells with \( \mathrm{H}_2 \mathrm{O}_2 \) were inhibited more significantly than those in the control group and that the cells in the \( \mathrm{H}_2 \mathrm{O}_2 \) group treated with the LZD-medicated serum proliferated faster than those in the \( \mathrm{H}_2 \mathrm{O}_2 \) group (Figure 1(c)).

3.2. Promotion of HUVEC Migration. To determine whether the LZD-medicated serum was capable of promoting HUVEC migration, cells were treated with different drug groups in a 2D migration assay. The results showed that HUVEC migration was significantly promoted by the treatment of LZD-medicated serum. Relative to that of the control group, the average distance of migration of the \( \mathrm{H}_2 \mathrm{O}_2 \) group cells to migrate was reduced to 25.33%. However, relative to that of the \( \mathrm{H}_2 \mathrm{O}_2 \) group, the migration distance of cells treated with \( \mathrm{H}_2 \mathrm{O}_2 \) and LZD-medicated serum increased to 66.67% (Figure 2).

3.3. Characterization of HUVECs and LZD-Enhanced Angiogenesis In Vitro. Tube formation is a key step for angiogenesis. HUVECs formed tubular structures in the Matrigel, and the effect was examined 24 h after being seeded. Exposure to the angiogenesis inhibitor \( \mathrm{H}_2 \mathrm{O}_2 \) resulted in the decrease in the capillary-like structures on the Matrigel. LZD treatment promoted the formation of tubular structures (Figure 3(d)). Fewer branch points were observed in the treated group with 400 \( \mu \mathrm{mol} / \mathrm{L} \) \( \mathrm{H}_2 \mathrm{O}_2 \) (Figure 3(c)) than in the control group (Figure 3(a)). No significant difference was found between the blank serum group (Figure 3(b)) and the control group. Quantitative measurements confirmed that exposure to LZD resulted in a significant increase in the mean number of branch points (Figure 3(e)). This result suggests that LZD may have a boosting effect on angiogenesis.

3.4. Production of Autophagosomes in HUVECs. To investigate whether \( \mathrm{H}_2 \mathrm{O}_2 \) could induce autophagy and to observe the effect of autophagy level with an LZD-medicated serum on HUVECs, we used TEM to detect autophagosomes. Autophagic vacuoles are morphological hallmarks of autophagy [20]. As shown in Figure 4, the control group (Figure 4(a)) and the cells were treated with blank serum (Figure 4(b)) for 24 h, and autophagosomes were not detected by TEM. Autophagic vacuoles containing cellular material or membranous structures in medically treated cells were detected.

3.5. Immunofluorescence Detection of LC3-II Expression. LC3-II plays an important role in the formation of autophagic vacuoles. It is considered a marker for the activation of
autophagosomes, and it is specifically localized in the double-layer membrane of autophagosomes. Thus, the immunofluorescence detection of LC3-II was used to determine the autophagic level. LC3-II expression was barely detectable in the control (Figure 5(A)) and blank serum groups (Figure 5(B)). In the experimental group, LC3-II was positive in HUVECs at 24 h (Figure 5(C)). The H$_2$O$_2$ pretreatment + LZD-medicated serum group showed a pattern of increased
LC3-II expression at 24 h (Figure 5(D)) and exhibited a more intense staining pattern than the H$_2$O$_2$ group. However, the LZD-medicated serum was shown to promote autophagy in HUVECs.

3.6. Expression of LC3-II and Beclin-1 Protein by Western Blot. To determine the effect of the LZD-medicated serum on cell autophagy of HUVECs, the expression of autophagy-related proteins LC3-II and Beclin-1 was examined using Western blot. The results showed that the conversion of LC3-I into LC3-II and Beclin-1 expressions increased after the LZD-medicated serum treatment compared with that of the H$_2$O$_2$ group (Figure 6(b)). No apparent expression of the autophagy-related protein was detected in the normal and blank serum groups (Figure 6(a)).

4. Discussion

IS is one of the major health issues in developing countries, and it leads to long-term morbidity and etiologies of mortality [21]. As previously mentioned, new vessel formation can provide therapeutic benefits in stroke management after IS. The underlying physiology of angiogenesis is complicated, as it involves the interaction of multiple signaling pathways and the expression of many growth factors, such as the vascular endothelial growth factor, transforming growth factors, platelet-derived growth factor, and basic fibroblast growth factor [22, 23]. Conventional angiogenesis studies usually focus on growth factors, but our previous experiments demonstrated that LZD could promote angiogenesis. In this study, experimental innovation was focused on the relevance between angiogenesis and cellular autophagy, and angiogenesis was explored from the perspective of cellular autophagy. The result shows that LZD-medicated serum promotes the migration, tube formation, and invasion of HUVECs accompanied by the upregulation of LC3-II and Beclin-1. This finding suggests that LZD may be a promising therapeutic drug to promote angiogenesis by improving cellular autophagy.

Autophagy occurs at a low basal level under physiological conditions in the body [24]. Autophagic elimination of injured mitochondria and endoplasmic reticulum splinters could contribute to this protection because of the termination of apoptosis [25, 26]. In addition, many studies have shown that the process of angiogenesis is accompanied by an increase in autophagy [27]. The main novelty of this study demonstrated that autophagy is necessary for therapeutic angiogenesis [28]. In this study, we investigated the role of autophagy in the process of angiogenesis and deduced that H$_2$O$_2$ was activated by reactive oxygen species to injure HUVECs through autophagy [29]. Conversely, H$_2$O$_2$ was used to intervene in HUVECs, and autophagy models were established. H$_2$O$_2$ induces autophagy and increased LC3-II and Beclin-1 expression [30, 31]. Autophagy proteins and autophagosomes increased when using LZD to repair the damaged HUVECs.

Cell migration plays a vital role in the process of angiogenesis, and it is the basis for assessing angiogenesis at the cellular level [32]. This dynamic process of angiogenesis is induced by cytokines, chemical substances, and external stimuli [33]. This study presented various experimental groups of cell migration through the wound scratch test. After the comparison and analysis of the experimental results, the LZD-medicated serum seems to stimulate the vitality of damaged cells and enhance their migration rate in vitro, which is beneficial to the angiogenesis of HUVECs and may increase the migrated cell number in the ischemic area. We also utilize the in vitro Matrigel to simulate the formation of a vascular lumen [34, 35], although the process of Matrigel-induced morphological differentiation of HUVECs is unlikely to be the same as the formation of new vessels, which occurs during angiogenesis in vivo. The in vitro Matrigel system in combination with a numerical analyzer allows the easy and rapid quantitative determination of tube formation induced by the Matrigel [36]. The model can still be used to define other factors or molecular events affecting new blood vessel formation with endothelial cell [37].

LC3-II and Beclin-1 are considered key regulators of autophagy in mammalian cells. However, little is known about LC3-II and Beclin-1 expression in endothelial cells. LC3-II, which serves as an autophagosome-specific protein [38], is one of the most reliable markers of autophagosomes
in mammals [39]. LC3-I exists in the cytoplasm, and LC3-II is a membrane-bound protein that attaches to autophagosomes and subsequently combines with lysosomes [40]. The amount of LC3-II reflects a large number of autophagosomes obtained through the process of LC3-I converting into LC3-II. Therefore, the induction and inhibition of autophagy could be monitored through the detection of LC3-II levels [41]. Cellular autophagic activity has been reported to occur in various types of cells [42]. Beclin-1 is strongly associated with the formation of autophagic membranes and may also be an important sign of autophagy [43]. Currently, Beclin-1 is usually studied in the field of tumors, including ovarian, breast, and prostate cancer [44]. In the present study, TEM demonstrated that autophagosomes increased with the LZD-medicated serum-induced new tube formation in HUVECs. In this process, Western blot analysis demonstrated that LC3-II and Beclin-1 expression increased, consistent with the result of fluorescence microscopy.

Figure 5: Immunofluorescence of LC3-II expression. HUVECs, human umbilical vein endothelial cells; LZDMS, long-zhi decoction medicated serum. (1) represents Nuclear staining; (2) represents cytoplasm staining; (A), (B), (C), and (D) represent a combination of nucleus and cytoplasmic staining. Immunofluorescence analysis showed extremely low LC3-II expression in the control group (A) and the blank serum group (B). The number of LC3-II positive HUVECs in the H_{2}O_{2} group (C), and the intensity of LC3-II staining increased with the treatment of LZD-medicated serum (D). The results were obtained at 24 h.
The debate on the effect of autophagy on the pathophysiology of cerebral ischemia remains unresolved. Mild-to-moderate autophagy has been proposed to encourage the survival of endothelium and that its overactivation promotes cellular death [45]. However, the current threshold for excessive autophagy is unclear [46]. Additionally, autophagic activities beneficial to vasculature cells may be involved in the protection of the integrity of angiogenesis. The effect of autophagy on the cell can be described as a double-edged sword [47]. \( \text{H}_2\text{O}_2 \) has been shown to induce autophagy above the basal level in HUVECs, as shown by a specific level of LC3-II and Beclin-1 [29]. Furthermore, the autophagy-related protein significantly improved when we added the LZD-medicated serum to protect the damaged cells and to promote HUVEC proliferation and tube formation. An increase in LC3-II may deter the completion of autophagy, and thus the autophagy shift can be assessed by measuring Beclin-1. The induction of an autophagic flux is usually accompanied by a high expression of LC3-II, but not of LC3-I, and increased amounts of Beclin-1.

5. Conclusion

In summary, the present result showed that the LZD-medicated serum promoted the proliferation, migration, and tube formation of HUVECs accompanied by the upregulation of LC3-II and Beclin-1. This finding suggests that LZD may be a promising therapeutic drug to improve brain function in IS. The autophagy of LZD-mediated serum-induced HUVECs may be involved in the process of new tube formation. The result further reveals that cellular autophagy has the potential to be a therapeutic strategy for IS and is a possible way to promote angiogenesis.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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References

[1] X. Zhang, X. Tang, K. Liu, M. H. Hamblin, and K.-J. Yin, “Long noncoding RNA malat1 regulates cerebrovascular pathologies in ischemic stroke,” The Journal of Neuroscience, vol. 37, no. 7, pp. 1797–1806, 2017.
[2] M. Ezzati, A. D. Lopez, A. Rodgers, S. Vander Hoorn, C. J. Murray, and G. Comparative Risk Assessment Collaborating, “Selected major risk factors and global and regional burden of disease,” The Lancet, vol. 360, no. 9343, pp. 1347–1360, 2002.
[3] N. Biswas and M. A. Sangma, “Serum LDL (Low Density Lipoprotein) As a Risk Factor for Ischemic Stroke,” Mymensingh Medical Journal, vol. 25, no. 3, pp. 425–432, 2016.
[4] L. Chen, C. Chang, L. Hsu et al., “Bacterial pneumonia following acute ischemic stroke,” Journal of the Chinese Medical Association, vol. 76, no. 2, pp. 78–82, 2013.
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[5] Q. Bai, Z. Lyu, Z. Pan, J. Lou, and T. Dong, “Epigallocatechin-3-gallate promotes angiogenesis via up-regulation of Nf2 signal transduction pathway in a mouse model of ischemic stroke,” *Behavioural Brain Research*, vol. 321, pp. 79–86, 2017.

[6] L. Ruan, B. Wang, Q. ZhuGe, and K. Jin, “Coupling of neurogenesis and angiogenesis after ischemic stroke,” *Brain Research*, vol. 1623, pp. 166–173, 2015.

[7] Q. Zhang and Y.-H. Zhao, “Therapeutic angiogenesis after ischemic stroke: Chinese medicines, bone marrow stromal cells (bmscs) and their combinational treatment,” *American Journal of Chinese Medicine*, vol. 42, no. 1, pp. 61–77, 2014.

[8] J. Liu, L. Fan, H. Wang, and G. Sun, “Autophagy, a double-edged sword in anti-angiogenesis therapy,” *Medical Oncology*, vol. 33, no. 1, p. 10, 2016.

[9] S. Kumar, S. K. Guru, A. S. Pathania, A. Kumar, S. Bhushan, and F. Malik, “Autophagy triggered by magnolol derivative negatively regulates angiogenesis,” *Cell Death & Disease*, vol. 4, no. 10, p. e889, 2013.

[10] S. J. Li, S. J. Sun, J. Gao, and F. B. Sun, “Wogonin induces Beclin-1/P13K and reactive oxygen species-mediated autophagy in human pancreatic cancer cells,” *Oncology Letters*, vol. 12, no. 6, pp. 5059–5067, 2016.

[11] A. Xu and G. Sweeney, “Emerging role of autophagy in mediating widespread actions of ADIPOQ/adiponectin,” *Autophagy*, vol. 11, no. 4, pp. 723–724, 2015.

[12] S. Song, J. Tan, Y. Miao, M. Li, and Q. Zhang, “Crosstalk of autophagy and apoptosis: Involvement of the dual role of autophagy under ER stress,” *Journal of Cellular Physiology*, vol. 232, no. 11, pp. 2977–2984, 2017.

[13] D. Denton, T. Xu, and S. Kumar, “Autophagy as a pro-death pathway,” *Immunology & Cell Biology*, vol. 93, no. 1, pp. 35–42, 2015.

[14] B. Sampaio-Marques, C. Felgueiras, A. Silva, F. Rodrigues, and P. Ludovico, “Yeast chronological lifespan and proteotoxic stress: Is autophagy good or bad?” *Biochemical Society Transactions*, vol. 39, no. 5, pp. 1466–1470, 2011.

[15] M. P. Tschan and H.-U. Simon, “The role of autophagy in anticancer therapy: Promises and uncertainties,” *Journal of Internal Medicine*, vol. 268, no. 5, pp. 410–418, 2010.

[16] H. Xiang, L. Zhang, J. Song et al., “The profiling and identification of the absorbed constituents and metabolites of Guizhi decoction in rat plasma and urine by rapid resolution liquid chromatography combined with quadrupole-time-of-flight mass spectrometry,” *International Journal of Molecular Sciences*, vol. 17, no. 9, 2016.

[17] D. Yin, Z. Liu, D. Peng et al., “Serum containing tao-hong-si-wu decoction induces human endothelial cell VEGF Production via PI3K/Akt-eNOS signaling,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 195158, 9 pages, 2013.

[18] Q. Zhang, Y. J. Yang, H. Wang et al., “Autophagy activation: a novel mechanism of avortastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway,” *Stem Cells and Development*, vol. 21, no. 8, pp. 1321–1332, 2012.

[19] H. Guo, Y. Chen, L. Liao, and W. Wu, “Resveratrol protects HUVECs from oxidized-LDL induced oxidative damage by autophagy upregulation via the AMPK/SIRT1 pathway;” *Cardiovascular Drugs & Therapy*, vol. 27, no. 3, pp. 189–198, 2013.

[20] H. Abeliovich, “Guidelines for the use and interpretation of assays for monitoring autophagy,” *Haematologica*, 2013.

[21] L. Rothstein and G. C. Jickling, “Ischemic stroke biomarkers in blood,” *Biomarkers in Medicine*, vol. 7, no. 1, pp. 37–47, 2013.

[22] T. Talwar and M. V. P. Srivastava, “Role of vascular endothelial growth factor and other growth factors in post-stroke recovery,” *Annals of Indian Academy of Neurology*, vol. 17, no. 1, pp. 1–6, 2014.

[23] R. G. Rubio and A. P. Adamis, “Ocular angiogenesis: Vascular endothelial growth factor and other factors,” *Developments in Ophthalmology*, vol. 55, pp. 28–37, 2015.

[24] X.-J. Li and S. Li, “Proteasomal dysfunction in aging and Huntington disease,” *Neurobiology of Disease*, vol. 43, no. 1, pp. 4–8, 2011.

[25] V. O. Kaminsky and B. Zhivotovsky, “Free radicals in cross talk between autophagy and apoptosis,” *Antioxidants & Redox Signaling*, vol. 21, no. 1, pp. 86–102, 2014.

[26] C. Lu, “Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis,” *Autophagy*, vol. 6, no. 7, p. 891, 2010.

[27] W. Lu, Y. Shi, Z. Ma et al., “Proper autophagy is indispensable for angiogenesis during chick embryo development,” *Cell Cycle*, vol. 15, no. 13, p. 1742, 2016.

[28] F. Wang, X. Dong, P. Xiu et al., “T7 peptide inhibits angiogenesis via downregulation of angiopoietin-2 and autophagy,” *Oncology Reports*, vol. 33, no. 2, pp. 675–684, 2015.

[29] M. Bai, R. Che, Y. Zhang et al., “Reactive oxygen species-initiated autophagy opposes aldosterone-induced podocyte injury,” *American Journal of Physiology-Renal Physiology*, vol. 17, no. 2, pp. F669–F678, 2015.

[30] G. Liu, X. Jiang, X. Zhu et al., “ROS activates JNK-mediated autophagy to counteract apoptosis in mouse mesenchymal stem cells in vitro,” *Acta Pharmacologica Sinica*, vol. 36, no. 12, pp. 1473–1479, 2015.

[31] C. Shu-rui, S. R. Meng-yao et al., “Methylprednisolone exerts neuroprotective effects by regulating autophagy and apoptosis,” *Neural Regeneration Research*, vol. 11, no. 5, pp. 823–828, 2016.

[32] Z. Y. Zhou, L. Y. Huan, W. R. Zhao, N. Tang, Y. Jin, and J. Y. Tang, “Spaltholobi Caulis extracts promote angiogenesis in HUVECs in vitro and in zebrafish embryos in vivo via up-regulation of VEGFRs,” *Journal of Ethnopharmacology*, vol. 200, pp. 74–83, 2017.

[33] Y. Hayon, O. Dashhevsky, E. Shai, A. Brill, D. Varon, and R. R. Leker, “Platelet microparticles induce angiogenesis and neurogenesis after cerebral ischemia,” *Current Neurovascular Research*, vol. 9, no. 3, pp. 185–192, 2012.

[34] M. L. Ponce, “Tube formation: An in vitro matrigel angiogenesis assay,” in *Methods in Molecular Biology*, vol. 467, pp. 183–188, Humana Press, 2009.

[35] C. P. Khoo, K. Micklem, and S. M. Watt, “A comparison of methods for quantifying angiogenesis in the matrigel assay in vitro,” *Tissue Engineering - Part C: Methods*, vol. 17, no. 9, pp. 895–906, 2011.

[36] C. Benedict and S. Vasanta, “Behavior of endothelial cells on Matrigel and development of a method for a rapid and reproducible in vitro angiogenesis assay,” in *In Vitro Cellular & Developmental Biology Animal*, vol. 43, pp. 87–94, 2007.

[37] R. Benelli and A. Albini, “In vitro models of angiogenesis: The use of Matrigel,” *The International Journal of Biological Markers*, vol. 14, no. 4, pp. 243–246, 1999.

[38] B. Levine and G. Kroemer, “Autophagy in aging, disease and death: The true identity of a cell death impostor,” *Cell Death & Differentiation*, vol. 16, no. 1, pp. 1-2, 2009.
[39] G. Talaber, G. Miklossy, Z. Oaks et al., "HRES-1/Rab4 promotes the formation of LC3+ autophagosomes and the accumulation of mitochondria during autophagy," PLoS ONE, vol. 9, no. 1, Article ID e84392, 2014.

[40] N. Zhang, L. Li, J. Wang et al., "Study of autophagy-related protein light chain 3 (LC3)-II expression levels in thyroid diseases," Biomedicine & Pharmacotherapy, vol. 69, pp. 306–310, 2014.

[41] D. A. Gewirtz, "The challenge of developing autophagy inhibition as a therapeutic strategy," Cancer Research, vol. 76, no. 19, pp. 5610–5614, 2016.

[42] P. Chai, H. Ni, H. Zhang, and X. Fan, "The evolving functions of autophagy in ocular health: A double-edged sword," International Journal of Biological Sciences, vol. 12, no. 11, pp. 1332–1340, 2016.

[43] L.-X. Ye, J. Yu, Y.-X. Liang, J.-S. Zeng, R.-X. Huang, and S.-J. Liao, "Beclin 1 knockdown retards re-endothelialization and exacerbates neointimal formation via a crosstalk between autophagy and apoptosis," Atherosclerosis, vol. 237, no. 1, pp. 146–154, 2014.

[44] K.-H. Eum and M. Lee, "Targeting the autophagy pathway using ectopic expression of beclin 1 in combination with rapamycin in drug-resistant v-Ha-ras-transformed NIH 3T3 cells," Molecules and Cells, vol. 31, no. 3, pp. 231–238, 2011.

[45] Y. Xie, S.-J. You, Y.-L. Zhang et al., "Protective role of autophagy in AGE-induced early injury of human vascular endothelial cells," Molecular Medicine Reports, vol. 4, no. 3, pp. 459–464, 2011.

[46] T. Kimura, Y. Takabatake, A. Takahashi, and Y. Isaka, "Chloroquine in cancer therapy: a double-edged sword of autophagy," Cancer Research, vol. 73, no. 1, pp. 3–7, 2013.

[47] J. Liu, L. Fan, H. Wang, and G. Sun, "Autophagy, a double-edged sword in anti-angiogenesis therapy," Medical Oncology, vol. 33, no. 1, p. 10, 2016.