Protective effect of Liangxue Huayu decoction on human retinal pigment epithelial cell (ARPE-19) injury induced by hypoxia through autophagy pathway

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Abstract

Background: Exploring the protective mechanism of the Liangxue Huayu (LXHY) decoction on human retinal pigment epithelial (RPE) cells induced by hypoxia through the autophagy pathway. Methods: The appropriate LXHY decoction concentration was determined by CCK-8. ARPE-19 cells were divided into the normal control group (A group), CoCl2 group (B group), 3-Methyladenine (3-MA) group (treated with 3-MA (the inhibition of autophagy pathway)) (C group), blank serum (BS) group (D group), LXHY drug-contained serum (DCS) group (E group), and Rapamycin (RAP) group (treated with LXHY drug-contained serum combined with rapamycin (the activation of autophagy pathway)) (F group). Counting the number of autophagosomes and autolysosomes in each group of cells under transmission electron microscopy. After infection of cells in each group by mRFP-GFP-LC3 fusion protein adenovirus, the strength of autophagic flux was detected. The mRNA expression levels of LC3 and Beclin-1 were detected by Q-PCR. Results: CCK-8 assay results showed that LXHY DCS could inhibit the cell proliferation of ARPE-19 under hypoxia (all P < 0.05). As the transmission electron microscopy assay result showed, compared with the normal control group, the number of autolysosomes was significantly increased in the CoCl2 group (P < 0.05). Compared with CoCl2 group, the number of autolysosomes was significantly reduced the 3-Methyladenine group, blank serum group, and LXHY drug-contained serum group (all P < 0.001). As autophagic flux assay result showed, compared with the normal control group, the level of autophagosomes and autolysosomes were significantly more than in CoCl2 group (all P < 0.001). Compared with the CoCl2 group, the level of autophagosomes and autolysosomes were significantly less than in the 3-Methyladenine group, blank serum group, and LXHY drug-contained serum group (all P < 0.05). The level of autolysosomes in the LXHY drug-contained serum group was lower than in the blank serum group (P < 0.05). Compared with the LXHY drug-contained serum group, the levels of autophagosomes and autolysosomes were significantly more than in the LXHY drug-contained serum group combined with rapamycin group (all P < 0.001). As the Q-PCR result showed, compared with the normal control group, the expression of LC3 and Beclin-1 mRNA were significantly reduced in the CoCl2 group (all P < 0.001). Compared with the CoCl2 group, the expression of LC3 mRNA were significantly increased in the 3-Methyladenine group, blank serum group, and LXHY drug-contained serum group (all P < 0.001). Beclin-1 mRNA expression was increase significantly (all P < 0.001) in the blank serum group and the LXHY drug-contained serum group. And Beclin-1 mRNA expression in the LXHY drug-contained serum group was statistically significant increased than blank serum group (P < 0.001). In the LXHY drug-contained serum combined with the rapamycin group, the LC3 and Beclin-1 mRNA expression was reduced significantly compared with the LXHY drug-contained serum group (all P < 0.001). Conclusion: The LXHY DCS has the ability to protect the human retinal pigment epithelial cell (ARPE-19) damage under hypoxia through the autophagy pathway.

Keywords: Liangxue Huayu decoction; age-related macular degeneration; autophagy; lysosome; autophagosomes
**Background**

Currently, age-related macular degeneration (AMD) is the leading cause of low vision and even blindness in the elderly, with AMD patients accounting for as high as 20.2% of the population aged over 70 in China. With the aging population, the number of AMD patients is steadily increasing [1]. Progressing AMD can be divided into geographic atrophy (GA) and neovascular AMD, also known as wAMD. wAMD is characterized by the formation of new blood vessels in the macula, which can lead to recurrent leakage, bleeding, and scar formation in the macular area, and it is one of the main causes of blindness in AMD patients.

Hypoxic conditions can diminish choroidal blood circulation. It also can stimulate hypoxiainducible factor-1 (HIF-1), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) that lead to RPE cell proliferation and contribute to neovascularization (NV). Besides, it can cause an increase in reactive oxygen species, resulting in oxidative stress damage to the retinal pigment epithelium (RPE) [2-4]. Some researchers have indicated that oxidative stress can lead to the occurrence of AMD by affecting RPE cell autophagic function, cellular aging, and immune inflammatory reactions. Impaired autophagic function in RPE cells can lead to lipofuscin deposition, drusen formation, and RPE damage or atrophy, thereby causing damage to photoreceptor cells and choroidal atrophy. Then, the damage worsens the progress of AMD [5].

The autophagic process involves the degradation of damaged, degenerated, or abnormally accumulated proteins, the removal of damaged mitochondria, excessive reactive oxygen species, and various pathogens. It helps maintain cell homeostasis. ATG6 (Beclin1) is an important switch for autophagic initiation. After binding to the intracellular membrane, it forms a pre-autophagosomal structure through the Atg12-Atg5-Atg16 complex. And the autophagosomal recruits microtubule-associated protein light chain 3 (LC3) to the membrane. After the complex dissociates, mature autophagosomes are formed. When cellular autophagy is initiated, LC3-I is processed and binds to phosphatidylethanolamine (PE) to form LC3-II, which is attached to the surface of the autophagosome membrane. LC3 can mediate the entry of substrates into the autophagosome, and finally complete degradation in the autolysosome [6]. In a word, Beclin1 is a key factor for autophagic initiation and can reflect the strength of autophagic activity. LC3 is a key molecule that regulates autophagy. Additionally, the levels of LC3 and Beclin1 can reflect the level of autophagic activity in cells and help evaluate autophagic activity levels [7, 8].

Currently, the main treatment for wAMD is intravitreal injection of anti-vascular endothelial growth factor (VEGF), but it is ineffective for some patients [1, 9]. In the previous research, our group found that the Liangxue decoction (LXHY) decoction was indeed effective in the treatment of wAMD, but its specific mechanism was still unclear and may be related to the autophagy pathway [10, 11].

In this study, CoCl2 will be used to induce hypoxic damage in ARPE-19 cells. In addition, the LXHY DCS would be an intervention condition to observe its protective effect on the hypoxic damage in ARPE-19 cells and its influence on the autophagic process. Thus providing an experimental and theoretical basis for the clinical application of the LXHY decoction in the treatment of AMD.

**Material and methods**

**Drugs and reagents**

The LXHY decoction used in the present study was provided by the TCM Pharmacy, the Second Affiliated Hospital of Fujian University of Traditional Chinese Medicine (Fujian, China), containing 15 g Pollen Typhae, 10 g Rhizoma Curcumae Longae, 15 g Herba Ecliptae, 15 g Practus Ligustri lucidi, 10 g Radix Angelicae Sinensis, 15 g Rhizoma Chuanxiong, 18 g Radix Salviae Miltiorrhiae, 15 g Practus Lycii, 20 g Radix Astragalii, 15 g Semen Cuscutae, 10 g Rhizoma Pinelliae, 10 g Bulbus Fritillariae Thunbergii. After being immersed in 1000 mL distilled water for 20 min and boiled for 45 min, the decoction of the herbal medicine was collected and condensed using a water bath at 100 °C, with a concentration of approximately 2.800 g/mL and diluted to 1.470 g/mL.

**Drug-contained serum preparation**

In total, 30 male Sprague Dawley (SD) rats obtained from HuFukang Biotechnology Co., Ltd. (Beijing, China) (SCXX (Beijing) 2019-0008) were randomly divided into two groups: the LXHY DCS group (20 rats) and the blank serum (BS) group (10 rats). LXHY decoction (1.470 g/100 g body weight) was administered to rats in the LXHY DCS group intragastrically for seven successive days, while equal volumes of physiological saline solution were administered to the rats in the BS group intragastrically over 7 days. Rat heart blood was collected 3 h after the last drug administration under strict aseptic conditions, and the serum was prepared by centrifugation at 2,500 rpm for 15 min. After being inactivated at 56 °C for 30 min, the serum was kept at −80 °C for cell treatments.

**Experimental animals**

SPF healthy male Sprague-Dawley rats (180 ± 20 g, 4 weeks) were purchased from HuFukang Biotechnology Co., Ltd. (Beijing, China) (SCXX (Beijing) 2019-0008). All rats were housed under standard laboratory conditions and fed specificopathogen-free quality food and purified water. The Animal Ethics Committee of the Institute of the Second People’s Hospital Affiliated to Fujian University of Traditional Chinese Medicine approved the animal experiments and the corresponding protocol [FJPSPH-IAEC2022063]. All experimental steps were performed in strict accordance with the guide for the care and use of laboratory animals (Eighth Edition. National Academies Press, Washington, USA). In addition, this study was conducted in accordance with the ARRIVE standard guidelines while implementing procedures to minimise test animals and potential hazards.

**Cell culture and treatment**

ARPE-19 cells obtained from the Shanghai Fuyu Biotechnology Co., LTD (Shanghai, China) were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (PAN biotech, German), and 1% penicillin and streptomycin (P/S) at 37 °C in a humidified cell culture supplied with 5% CO2. While cell confluency reached approximately 80%, the cells were treated with drugs. In addition to the normal control group (A group), all the remaining groups (B-F groups) of cells were exposed to a process known as hypoxia modeling, which involved the use of CoCl2 (232696-5G, Merck) 48 h. ARPE-19 cells in C group were treated with 3-Methyladenine (3-MA) (the inhibition of autophagy pathway), BS in D group, LXHY DCS in E group, and LXHY DCS combined with Rapamycin (RAP) (the activation of autophagy pathway) in F group (RAP were used after modeling for 4 h and before LXHY DCS treating). After different treatments for 24 h, cells were collected for the following assays.

**Drug concentration screening with CCK-8**

1 × 10^4 cells per well were plated in 96-well plates and treated with CoCl2 at concentrations of 200 umol/L 48 h. After hypoxia modeling with CoCl2, cells were treated with LXHY DCS at volume fractions of (0, 3.125, 6.25, 12.5, 25, 50, 100%) for 24 h. The cell viability was determined using CCK-8 (G6005M, USEVERBRIGHT).

**Autophagic flux observation**

After infection with mRFP-GFP-LC3 fusion protein adenovirus, the monomeric red fluorescent protein (mCherry) is used to label LC3B in the green fluorescent protein (GFP) and mCherry fusion protein. The fusion of GFP and mCherry fluorescent proteins appears as yellow dots. When autophagosomes fuse with lysosomes to form autolysosomes, the acidic lysosomal environment quenches the acid-sensitive GFP fluorescence, while mCherry remains unaffected, resulting in red fluorescence in autolysosomes. The counting and
photography of different colored dots reflect the strength of autophagic flux. Additionally, an increase in red dots indicates an elevated level of autophagic flux [12].

**Autophagosome under electron microscopy examination**

ARPE-19 cells were fixed in 2.5% glutaraldehyde (Spi-Chem, America), then embedded in low-temperature agarose, followed by secondary fixation, dehydration, infiltration, embedding, polymerization, sectioning, staining, and counting the number of autophagosomes and autolysosomes in each group of cells under transmission electron microscopy (HITACHI, HT7700, Japan). Lastly, images were collected and analyzed [12].

**Q-PCR**

Q-PCR was used in the present study to determine gene mRNA levels in ARPE-19 cells following a previously described method. Cells were collected, washed with PBS, added Trizol reagent, extracted total RNA, identified RNA purity and concentration, added the total RNA into cDNA, added primers and reaction reagent according to the kit, fluorescence quantitative PCR detection (Archimed X6, Kunpeng Gene Technology Co., Ltd. (Beijing, China). Reaction conditions: 95 °C 10 s, 60 °C 30 s, 40 cycles. GAPDH was set as an internal reference, and the normal group was set as 1, and 2^(-ΔΔCt) was used to reflect the relative mRNA expression level of the target gene. LC3: F: 5AACATGAGCGAGTTGGTCAAG, R: 5GCTGCTAGATGTCGCCGAT3; Beclin-1: F: 5GTTGCTCTTGCGAGTTGGAAG, R: 5GTCGCCCATCATCCT3; 3-Methyladenine (3-MA): F: 5GTGGTCTCGGCTAGGATC3; GAPDH: F: 5GTTGCTACCATCATCCT3; R: 5GAGTCTTCACGATAGCAAG3. Table 1 shows the primer sequences.

**Statistical analysis**

All data were presented as the means ± standard deviation (SD). The results were analyzed by one-way analysis of variance (ANOVA) or the Student t-test, and the LSD-t test was applied to analyse between two groups. P < 0.05 was considered to be statistically significant.

**Result**

**LXHY DCS could inhibit the cell proliferation of ARPE-19 under hypoxia**

It can be seen in Figure 1 that at LXHY DCS at 6.25%, 25%, 50% and 100% volume fraction could decrease ARPE-19 activity under hypoxia at 24 h. Only a 100% volume fraction could decrease the activity in hypoxia at 48 h. Different volume fraction of LXHY DCS could reduce the activity under hypoxia at 72 h. The above differences were statistically significance (all P < 0.05). Therefore, LXHY DCS could inhibit the cell proliferation of ARPE-19 under hypoxia. And for further experiment, 6.25% of LXHY DCS were chosen to explore the mechanism of its protection for ARPE-19 under hypoxia.

**LXHY DCS reduced the autolysosomes counts of ARPE-19 cells under hypoxia**

Transmission electron microscopy showed that there was no significant difference in autophagosomes between the groups (P = 0.535). Statistical differences of autolysosomes were observed in different groups (P < 0.001). Under hypoxia, the count of autolysosomes of ARPE-19 cells increased significantly (LSD-t = 2.757, P < 0.05). Compared with the B group, the count of autolysosomes in the C group, D group, and E group significantly reduced (LSD-t= 4.097, LSD-t= 4.182, LSD-t=LXHYS= 5.402, all P < 0.001). And there was no significant differences between D group and E group (LSD-t = 0.888, P = 0.379). Compared with the E group, the count of autolysosomes increased in the F group, but the difference was not significant (LSD-t = −0.503, P = 0.617). As shown in Figure 2 and Figure 3.

**Figure 1** LXHY DCS volume fractions screening with CCK-8. Compared with normal ARPE-19 cells, P < 0.05. LXHY, Liangxue Huayu.

**Figure 2** ARPE-19 cells in each group were observed under transmission electron microscopy. (A) Normal group. (B) Model group. (C) 3-Methyladenine (3-MA) group. (D) Blank serum (BS) group. (E) Liangxue Huayu drug-contained serum (LXHY DCS) group. (F) LXHY DCS combined with Rapamycin (RAP) group.
**Figure 3** Effect of LXHY DCS on autophagosomes and autolysosomes of ARPE-19 cells under hypoxia. (A) Normal group. (B) Model group. (C) 3-Methyladenine (3-MA) group. (D) Blank serum (BS) group. (E) Liangxue Huayu drug-contained serum (LXHY DCS) group. (F) LXHY DCS combined with Rapamycin (RAP) group. ‘Compared with A group, P < 0.05.’ Compared with B group, P < 0.05.

**LXYH DCS decreased the count of LC3 dots in the autophagic flux of ARPE-19 cells under hypoxia**

Compared with the A group, the yellow dots increased in the B group. The difference was statistically significant (LSD-t = −5.187, P < 0.001), suggesting that the level of autophagosomes was risen. Compared with the B group, the yellow dots reduced in the C group and E group. The differences were statistically significant (C: LSD-t = 3.691, P < 0.001; E: LSD-t = 2.894, P < 0.05), which suggested the level of autophagosomes fell down. The yellow dots decreased in the D group. The difference was not statistically significant (LSD-t = 1.535, P = 0.128). Compared with the D group, the yellow dots were reduced in the E group, and the difference was not statistically significant (LSD-t = 1.360, P = 0.177). In comparison with the E group, the yellow dots increased in the F group. The difference between the two groups was statistically significant (LSD-t = −7.051, P < 0.001), indicating the levels of autophagosomes were risen, as shown in Figure 4B.

Compared with the A group, the red dots increased in the B group. The difference was statistically significant (LSD-t = −6.416, P < 0.001), suggesting that the level of autolysosomes rose. Compared with the B group, the red dots reduced in the C group, D group, and E group, the differences were statistically significant (C: LSD-t = 5.830, P < 0.001; D: LSD-t = 3.666, P < 0.001; E: LSD-t = 6.314, P < 0.001), which suggested the level of autolysosomes fell down. Compared with the D group, the red dots were reduced in the E group. The difference was statistically significant (LSD-t = 2.648, P < 0.05).

In comparison with the E group, red dots increased in the F group. The difference between the two groups was statistically significant (LSD-t = −2.470, P < 0.05), indicating the levels of autolysosomes had risen. As shown in Figure 4C.

**LXYH DCS enhanced the level of LC3 and Beclin-1 mRNA of ARPE-19 cells under hypoxia**

Compared with the A group, a significant reduction of LC3 and Beclin-1 mRNA expression happened in the B group. The difference was statistically significant (LC3: LSD-t = 42.842, P < 0.001; Beclin-1: LSD-t = 8.129, P < 0.001). Compared with the B group, significant increasing LC3 mRNA expression appeared in the C group, the D group and the E group, the differences were all statistically significant (LSD-t = −5.621, LSD-t = −5.339, LSD-t = −6.907, all P < 0.001). Beclin-1 mRNA expression in the D group and the E group increased greatly. The differences were all statistically significant (LSD-t = −15.361, LSD-t = −20.067, all P < 0.001). The increase of Beclin-1 mRNA expression in the C group wasn’t statistically significant (LSD-t = −0.679, P = 0.512). There was no significant difference between D group and E group in LC3 mRNA expression (LSD-t = −1.568, P = 0.143). And the expression of Beclin-1 mRNA in the E group was more significantly increasing than that in the D group (LSD-t = −6.328, P < 0.001). In the F group, LC3 and Beclin-1 mRNA expression reduced significantly compared with the E group (LC3: LSD-t = 5.617, P < 0.001; Beclin-1: LSD-t = 10.232, P < 0.001). As shown in Figure 5.

**Discussion**

Autophagy plays a central role in the regulation of cell homeostasis during AMD. The main cellular factor leading to AMD is the interruption of the lysosomal clearance process and waste accumulation. The weakening of autophagic clearance is an important mechanism in the pathogenesis of AMD [6, 13]. Chen Yunzhen et al. found that improving cellular autophagic activity could reduce the damage of RPE cells to some extent and inhibit the secretion of inflammatory factors and angiogenic factors in ARPE-19 cells [14]. Therapeutics targeting the enhanced autophagy pathway have the potential to treat AMD [15]. However, some scholars have said that autophagy may play a dual role in retinal degenerative diseases. Appropriate enhancement of autophagy during AMD lesions may be beneficial, but the autophagy may lead to retinal cell death and inhibit protective effects when overenhanced [16]. The autophagic process involves the fusion of autophagic vesicles, binding to LC3 to form the autophagosome, the autolysosome phagocytosis of damaged organelles and protein aggregates, and the transport to the lysosome.
Figure 4 Effect of LXHY DCS on the autophagic flux in hypoxic-injured ARPE-19 cells. (A) Normal group. (B) Model group. (C) 3-MA group. (D) BDCS group. (E) LXHY DCS group. (F) LXHY DCS combined with RAP group. * Compared with A group, P < 0.05. △ Compared with B group, P < 0.05. ▲ Compared with C group, P < 0.05. ▶ Compared with D group, P < 0.05. ▼ Compared with E group, P < 0.05.

Figure 5 Effect of LXHY DCS on the mRNA expression of LC3 and Beclin-1 in hypoxic ARPE-19 cells. (A) Normal group. (B) Model group. (C) 3-MA group. (D) BDCS group. (E) LXHY DCS group. (F) LXHY DCS combined with RAP group. * Compared with A group, P < 0.05. △ Compared with B group, P < 0.05. ▲ Compared with C group, P < 0.05. ▶ Compared with D group, P < 0.05. ▼ Compared with E group, P < 0.05.
to form autolysosomes for the final degradation [17]. This study found that both autophagosomes and autolysosomes were increased in ARPE-19 cells under hypoxia, promoting autophagic flux generation. The presence of autophagosomes and autolysosomes was also found under transmission electron microscopy, indicating that hypoxia can affect the autophagic process in ARPE-19 cells. The above study findings are similar to those of Rong Li [18]. We further examined the mRNA expression of LC3 and Beclin-1 and found that they were decreased under hypoxia. The expression of LC3 and Beclin-1 mRNA was instead higher under 3-MA with this phenomenon related to the negative feedback effect of the cell itself.

Our team used LXHY decoction to supplement the liver and kidney, in line with the sick machine, and related clinical studies found that it was effective in the treatment of wAMD [5, 19]. This study found that it not only inhibited the proliferation of ARPE-19 cells under hypoxia but also reduced the levels of autophagosome and autolysosomes in ARPE-19 cells under hypoxia. It also decreased the number of autolysosomes under transmission electron microscopy. Additionally, we found that the expression of LC3 and Beclin-1 mRNA was up-regulated, similar to the autophagic inhibitor group.

Previous studies showed that rapamycin could activate autophagic in cells under hypoxia, promote autophagic flux expression and enhance the expression of Beclin-1 and LC3 [20, 21]. This study found that after rapamycin pretreatment, the effect of Liangxue Huaya decoction in inhibiting autophagic flux was weakened, and the expression of LC3 and Beclin-1 mRNA was reduced, which indicates that the results of mRNA levels are inconsistent with autophagic flux and the number of subcellular organelle. The phenomenon may be caused by the negative feedback of the body.

In this study, it was found that the LXHY decoction protected against ARPE-19 by affecting the autophagic process of ARPE-19 under hypoxic conditions. However, there was still insufficient relevant research. On the one hand, this study is only an in vitro experiment, and the autophagic level is easy to change by the influence of environment, experiment, and operation. On the other hand, the pathogenesis of wAMD is complex. It is difficult to clarify whether LXHY decoction can protect hypoxia-damaged RPE cells through the autophagy pathway in patients. Therefore, the effects of LXHY decoction therapy on the autophagic process of RPE cells in patients can be deeply explored in future studies.

### Conclusion

The LXHY DCS has the ability to protect the human retinal pigment epithelial cell (ARPE-19) damage under hypoxia through the autophagy pathway.

### References


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