

# P53/DRAM/ autophagy - a new target for improving the therapeutic effect of anti-VEGF on intraocular neovascularization

**Yi Wang**

Center of Translational Medicine, Xi'an Ninth Hospital Affiliated to Medical College of Xi'an Jiaotong University

**Yao Yang**

Department of Ophthalmology, The first Affiliated Hospital of Xi'an Medical University

**Rong Li**

Department of Ophthalmology, The first Affiliated Hospital of Xi'an Medical University

**Binghui Wu**

Department of Ophthalmology, Xi'an No.1 Hospital

**Huiqin Lu**

Department of Ophthalmology, Xi'an No.1 Hospital

**Jing Cheng**

Department of Ophthalmology, Xi'an Ninth Hospital Affiliated to Medical College of Xi'an Jiaotong University

**Zhe Liu**

Center Translational medicine, Xi'an Ninth Hospital Affiliated to Medical College of Xi'an Jiaotong University

**Junhui Du** (✉ [djh79918@163.com](mailto:djh79918@163.com))

<https://orcid.org/0000-0001-6692-3877>

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## Research

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# Abstract

## Background

Recurrence of intraocular neovascularization is a major clinical problem. Anti-VEGF drugs are the main treatment for intraocular neovascularization currently. However, anti-VEGF drugs can activate endothelial autophagy and weaken the therapeutic effect. This study aims to elucidate the effect and mechanism of anti-VEGF drugs on autophagy of vascular endothelial cells.

## Methods

RF/6A cells were randomly divided into five groups: The control group, hypoxia group (1% O<sub>2</sub> / 5% CO<sub>2</sub> / 94% N<sub>2</sub>), anti-VEGF group (group1: Ranibizumab 100ug/ml; group2: Aflibercept, 400ug/ml; group3: Conbercept, 100ug/ml) and autophagy inhibition group (3-MA or CQ) which was corresponding to anti-VEGF group. Autophagy-related proteins were examined by Western blot. RFP-GFP-LC3 was used to detect autophagy and autophagic flow. CCK-8 assay was used to detect cell proliferation. Flow cytometry and TUNEL was used to detect cell apoptosis. Cell migration and tube formation were assessed by wound assay and matrix method, respectively.

## Results

Ranibizumab and Conbercept can trigger autophagy in hypoxia condition in RF/6A cells, while Aflibercept can inhibit autophagy. Conbercept combined with autophagy inhibitor (3-MA or CQ) could inhibit cell migration and tube formation of RF/6A cells more effectively in hypoxia condition. For mechanism, p53 and DRAM proteins play an important role in Conbercept induced autophagy. Inhibition of P53 can suppress the autophagy induced by Conbercept.

## Conclusion

Ranibizumab and Conbercept can trigger the autophagy of vascular endothelial cells while Aflibercept can inhibit it. The combination of ranibizumab/ Conbercept and autophagy inhibitor can significantly inhibit the formation of angiogenesis in vitro. The mechanism of autophagy activation is related to the activation of p53 / DRAM pathway.

## Background

The formation of intraocular neovascularization is main cause of ophthalmological visual loss caused by age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), central retinal vein occlusion (CRVO), and other eye diseases[1–3]. At present, the main treatment is anti-vascular endothelial growth factor (anti-VEGF) in the treatment of intraocular neovascularization. Although anti-VEGF therapy has achieved good therapeutic effect, the recurrence of neovascularization is inevitable which increases the cost of treatment and adverse drug reactions, and also increase the incidence of complications such as endophthalmitis, lens damage, elevated intraocular pressure[4, 5]. Therefore, How to control the

formation of intraocular neovascularization more effectively and reduce the recurrence of intraocular neovascularization is particularly important.

In recent years, it has been found that anti-angiogenic therapy could increase the level of autophagy in tumor cells[6, 7]. It is reported that inhibition of autophagy in liver cancer cells under hypoxic culture could reduce cell viability and promote apoptosis[8]. Other studies have found that the combination of vascular inhibitors and autophagy inhibitors could significantly inhibit tumor growth, promote the apoptosis of tumor cells[9]. In addition, the combination of autophagy inhibitors has been proved to be more effective in controlling the growth of tumor growth[10, 11]. The mechanism of tumor cells self-protection in anti-angiogenic therapy and anti-vascular drug resistance may be that autophagy has the function of clearing degenerative aging proteins and damaged organelles, which is helpful to maintain the homeostasis of the intracellular environment and help cell through the dangerous phase.

Previous study have found that Ranibizumab, Bevacizumab and other anti-tumor angiogenic inhibitor could induce autophagy[12–14]. However, the relationship between anti-VEGF therapy and autophagy and its role in the recurrence of intraocular neovascularization remains unclear. The purpose of this research is to investigate whether anti-VEGF therapy can activate autophagy of choroidal vascular endothelial cells and the underlying mechanism.

## Methods:

## Reagents

Rhesus choroid-retinal endothelial cells (RF/6A cells) were purchased from Type Culture Collection of Chinese Academy of Sciences. Ranibizumab/Lucentis was purchased from Novartis Pharma Stsin AG, Aflibercept was obtained from Sanofi and Regeneron Pharmaceuticlas, Inc(Lot# 4F002A), Conbercept from Chengdu kanghong Pharmaceutical Group co., Ltd ( Sichuan, China). Beclin-1 was purchased from Bioword technology, MN, USA. 3-methyl adenine(3-MA) and p53 inhibitor [Pifithrin- $\alpha$  (PFT $\alpha$ ) HBr] (Selleck, USA) and Chloroquine (CQ) was purchased from Aladdin (USA).

## Cell Culture and Treatment

The RF/6A cells were incubated in RPMI-1640 medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humidified 95% room air with 5% CO<sub>2</sub>. RF/6A cells were divided into the following groups: control group(RF/6A were cultured under normal oxygen conditions), hypoxia group( 1% O<sub>2</sub> 5% CO<sub>2</sub> 94% N<sub>2</sub> ), anti-VEGF group (group1: Ranibizumab 100ug/ml; group2: Conbercept, 100ug/ml; group3: Aflibercept, 400ug/ml), autophagy inhibition group (group1: 3-MA + Conbercept, RF/6A cells were pretreat with 5 mM 3-MA for 1.5 h; group2: CQ + Conbercept, RF/6A cells were pretreat with 20  $\mu$ M CQ for 2.0 h).

## Cell activity assay

Cell Counting Kit (CCK8) assay was performed to measure the cell viability. Cells were cultured in 96-well plates at a density of 5000 cells and received the described treatments. 10 $\mu$ L CCK8 (Beyotime Institute of Biotechnology, Jiangsu, China) were added to each well and incubated for 4 h at 37°C. The absorbance value was then determined at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

## Cell migration assay

The migration ability of cells was tested by wound healing test. RF/6A cells with a density of  $1 \times 10^5$ /ml were added to 6-well plates, scratches were made using a pipette tip (200  $\mu$ l). Then, cells were flushed three times with PBS, and subjected to the RPMI-1640 for 36 h. Images were taken by microscope (Olympus IX50, Tokyo, Japan) at 0 and 36 h. The relative migration distance was measured by the Photoshop image analysis software. Three independent experiments were performed.

## Cell tube formation assay

The Matrigel Matrix was poured into 96-well plates (100  $\mu$ l/ well) and incubated for 30 minutes at 37 °C. RF/6A cells were added onto the layer of matrigel ( $2 \times 10^4$  cells per well) in RPMI-1640 medium containing or absence of various drugs. After 24 h of incubation, the cells in five randomly fields were photographed using an inverted microscope (Olympus IX50, Tokyo, Japan). The number of tube-like structures was measured and analyzed by Image J software (NIH, USA). Every group was designed to set three parallel holes and each experiment was repeated three times.

## TUNEL assay

TUNEL was carried out to evaluate cell apoptosis according to the manufacturer protocol. Induced apoptotic cells were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) at room temperature for 30 min, washed with PBS 3 times and then incubated on ice using 0.1% Triton X-100. After that, the treated cells were mixed with TUNEL reaction mixture, followed by reaction in the dark for 1 h at 37 °C. The number of apoptotic cells was obtained. It is used to reflect the severity of apoptosis in lung epithelial cells and lung endothelial cells.

## Flow cytometry analysis

Flow cytometry was performed for further analysis of cell apoptosis. RF/6A cells were grown in six-well culture plates were harvested using trypsin and washed in PBS. Then, the cells were resuspended in Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) and added with 5 ml Annexin V-FITC (BMS147FI; eBioscience, San Diego, CA, USA) for 20 min and after with 5 ml PI at room temperature for additional 15 min in the dark. The cells were acquired by flow cytometer (BD Biosciences) within 1 h after staining. At least 3,000 events were collected, and the data were analyzed by Cell-Quest Pro software (BD Biosciences).

## Autophagic flux assay

Tandem sensor RFP-GFP-LC3 constructs when cells grown on 12-well plates reached 40–50% of confluence, they were transfected with 50  $\mu$ L adenovirus expressing mCherry-GFP-LC3B fusion protein (Ad-mCherry-GFP-LC3B; Beyotime) at a multiplicity of infection (MOI) of 25 in 1640 medium together with indicated

treatment for 48 h at 37°C. After that, autophagy flux was observed under a laser confocal microscope (C2, Nikon, Tokyo, Japan). Then, They were transfected into RF/6A cells by a transient transfection method using Lipofectamine 2000 (Invitrogen) and exposed to 20  $\mu$ M Tan I for 24 h. Then, the red and green channel signals were observed in Tan 1-treated RF/6A cells under the Delta Vision imaging system (Applied Precision). Additionally, Tan I (20  $\mu$ M)-treated cells were co-incubated with 3 mM NH<sub>4</sub>Cl (Sigma) and 1 mM 3MA (CALBIOCHEM) for 3 h and observed under a FLUOVIEW FV10i confocal microscope (Olympus). Also MTT assay was conducted in H28 cells after Tan 1 treatment for 24 h with or without 3 mM NH<sub>4</sub>Cl (Sigma) and 1 mM 3MA (CALBIOCHEM).

## Western Blot Assay

We measured protein concentrations by biocinchoninic acid kits (BCA, Beyotime). Through 10% and 12% SDS-PAGE electrophoresis, and then transferred to the PVDF membrane. The membranes were blocked in 1% BSA for 1 h, then incubated with primary antibodies for 18 h at 4 °C. LC3(1:1000, CST, ), Atg7 (1:1000, CST), Beclin-1 (1:1000, Bioworld), p62 (1:2000, Abcam) P53 (1:2000, Abcam), DRAM(1:500, Novus), LAMP1(1:1000, Abcam) and GAPDH (1:1000, Bioworld) as loading control, then washed three times and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotech, CA, USA) for 1 hour. After being rinsed three times by TBST, The specific bands were visualized with enhanced chemiluminescence reagent following the manufacturer's instructions, and quantified using a chemiluminescence imaging system (CliNX, Shanghai, China). All experiments were repeated three times.

## Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software program. Data from several experiments were pooled and subsequently presented as mean  $\pm$  standard deviation, SNK method was used to make comparisons between pairs of groups, a two-tailed p-value of  $P < 0.05$  was considered statistically significant.

## Results

### 1. The influence of anti-VEGF on autophagy in RF/6A cells under hypoxia condition

Ranibizumab, Conbercept and Aflibercept are the most commonly used angiogenesis inhibitor in treatment of intraocular neovascularization. We used these three anti-VEGF drugs to investigate the effect of autophagy on RF/6A cells, the protein levels of autophagy markers LC3, Beclin-1, Atg7 and p62 were measured by western blotting after 24 h of cell culture. As shown in (Fig. 1), Compared with hypoxia

group, the protein level of Beclin-1 and LC3-2/1 in Ranibizumab and Conbercept groups were significantly higher ( $P < 0.05$ ). While the expression of P62 were decreased ( $P < 0.05$ ). The autophagic fluxare showed the same results (Fig. 2). In the case of autophagy, the number of yellow punctaes in Ranibizumab and Conbercept groups was more than that in hypoxia group and the red spots are increased when autophagy fused with lysosome. These results indicated that autophagy occurred in Ranibizumab and Conbercept groups. These results suggest that Ranibizumab and Conbercept can triger autophagy of RF/6A cells in hypoxia conditions. However, Aflibercept had a different effect on autophagy which inhibited the expression of Beclin-1 and LC3-2/1.

## **2. Effects of Conbercept combined with autophagy inhibitor on cell proliferation**

In order to further verify the interaction between autophagy and anti-VEGF inhibitors, we chose Conbercept as the representative to do further research. The Cell activity was analyzed by CCK8. As shown in Fig. 3. Compared with control group. Cell proliferation rate was decreased in hypoxia group 85.34% and conbercept group 79.97% ( $P < 0.05$ ). Autophagy inhibitor 3-MA or CQ can further inhibit cell proliferation (75.41%Vs 73.66) ( $P < 0.05$ ).

## **3. Effects of Conbercept combined with autophagy inhibitor on cell apoptosis**

The cell apoptosis were performed by flow cytometry and Tunel. The flow cytometry analysis showed that compared with control group, Hypoxia increased the apoptosis of RF/6A cells( $2.23 \pm 1.25$  Vs  $3.62 \pm 1.31$ ). Compared with hypoxia group, the apoptosis rate of RF / 6A cells in Hypoxia + Conbercept, Hypoxia + Conbercept + 3-MA,

Hypoxia + Conbercept + CQ groups increased in turn. ( $6.48 \pm 1.13$  Vs  $11.68 \pm 2.24$  Vs  $15.05 \pm 2.50$ ) (Fig. 4). The result of TUNEL was showed in Fig. 5. Blue color is the standard nucleus, while red is the nucleus labeled with tunle, which is used to represent apoptotic cells. There results indicated that inhibition of autophagy in RF/6A cells under hypoxic culture could promote apoptosis. The nuclear morphology of the control group was complete, indicating that there was no apoptosis. In hypoxia group, there were red labeled positive nuclei, indicating that apoptosis increased. In the group of Hypoxia + Conbercept, Hypoxia + Conbercept + 3-MA and Hypoxia + Conbercept + CQ, the number of red nuclei increased gradually. The results are consistent with those of flow cytometry.

## **4. Conbercept combined with autophagy inhibitor on cell migration and tube formation**

The effects of RF/6A cells migration and tube formation were detected by scratch wound test and Matrigel assay, respectively. Results indicated that after 36 h of cell incubation, a large number of cells migrated into the bare area of the cell culture plates (Fig. 6). Conbercept significantly inhibited cell migration compared with hypoxic group( $633.083 \pm 72.52$  Vs  $546.33 \pm 24.61$ ), while autophagy inhibitor group (3-MA or CQ) had more obvious inhibition effect( $309.75 \pm 86.36$  and  $263.33 \pm 68.67$ ) ( $P < 0.05$ ). For tube formation, the number of tube formation was decreased significantly in conbercept group ( $30 \pm 2.23$ ) compared to hypoxia group( $36 \pm 2.2$ ) and even further reduced in autophagy inhibitor group ( $27 \pm 3.42$ ).

The length of master segments in hypoxia group was higher than that in control group. ( $14641.33 \pm 1512.3$  Vs  $9056.67 \pm 423.22$ ). Compared with hypoxia, master segments length were decreased in Conbercept ( $14242.67 \pm 942.34$ ) and Conbercept + 3-MA group ( $12430.33 \pm 567.32$ ) (Fig. 7). All these results demonstrated that conbercept combined with autophagy inhibitor (3-MA) could inhibit cell migration and tube formation of RF/6A cells in hypoxia condition more significantly.

## 5. The effect of concept on the expression of p53 / DRAM

The expression of p53 and DRAM were tested by western blot and qPCR. As shown in Fig. 8,9. The expression of p53 and DRAM were increased in Conbercept and Hypoxia group ( $P < 0.05$ ). In order to further clarify the mechanism of Conbercept activated autophagy, the effect of Pifithrin- $\alpha$  (PFT $\alpha$ ) HBr on autophagy related proteins was analyzed. The results showed that compare with hypoxia group, the expression of LC-3, LAMP1, Beclin-1 and DRAM were increased in Conbercept group ( $P < 0.05$ ). However, this trend was attenuated in Pifithrin- $\alpha$  (PFT $\alpha$ ) HBr group ( $P < 0.05$ ) (Fig. 10). It shows that inhibiting p53 can inhibit the expression of DRAM and autophagy related proteins.

## Discussion

Intraocular neovascularization is a common pathological feature of various ischemic retinopathy[15, 16]. Although the occurrence of neovascularization is a protective response of the body, which plays an important role in tissue repair and ischemia-reperfusion[17, 18], its secondary lesions such as intraocular hemorrhage, retinal proliferation, traction retinal detachment and neovascular glaucoma can lead to loss of vision[19, 20]. In order to control intraocular neovascularization more effectively, Clinically, antiangiogenic drugs have become a powerful weapon in the treatment of neovascularization, especially choroidal neovascularization, the researchers even taken a variety of combined treatment, such as anti-VEGF drugs combined with photodynamic therapy, anti-VEGF drugs combined with glucocorticoid therapy, but the recurrent neovascularization problem can not be solved effectively. Therefore, other mechanisms of neovascularization are worth exploring. In our previous studies, we have found that anti-VEGF can activate autophagy in vascular endothelial cells, and the weakening of antiangiogenic effect may be related to the activation of autophagy[21–23]. However, the detailed mechanism is not clear. Therefore, we use three commonly used antiangiogenic drugs (Ranibizumab, Aflibercept and Conbercept) and autophagy inhibitors to explore the role and mechanism under recurrent choroidal neovascularization

In this study, we found that Ranibizumab and Conbercept could induce autophagy in RF/6A cells under hypoxia, while Aflibercept can inhibit it. Our previous study found that the activation of autophagy in turn can promote angiogenesis, thus weakening the role of antiangiogenic drugs. Therefore, induced autophagy may be the cause of poor therapeutic effect of antiangiogenic drugs. To verify this assumption, 3-MA and Chloroquine were used. They are autophagic inhibitors that act on different stages of autophagy. We founded that Conbercept combined with 3-MA or Chloroquine could suppressed cell proliferation, migration and tube formation more significantly and promoted apoptosis of RF/6A cells under hypoxia condition. This result is consistent with other researchs which found that combined

Bevacizumab (an Anti-VEGF drug) and autophagy inhibitor could significantly inhibit tumor growth and promote apoptosis in hepatocellular cancer and malignant glioma.[9, 24] All the above studies indicated that autophagy can be used as a new target for improving the therapeutic effect of angiogenesis inhibitors in the treatment of neovascular diseases. Interestingly, in our study, we found that Aflibercept had a different effect on autophagy. Previous studies and mechanisms cannot explain the longer and better therapeutic effect of Aflibercept in clinical treatment. We speculate that the mechanism may be related to inhibiting autophagy.

Damage-regulated autophagy modulator (DRAM) is a lysosomal protein involved in P53-regulated autophagy induction[25, 26]. Studies have found that DRAM leads to increased expression of beclin1 and production of p53[27]. Gao Z found that neuronal apoptosis induced by Nucleoside analog correlates with p53 overexpression and that the p53 target gene DRAM is involved[28]. Studies also found that p53/DRAM signaling pathway appears to contribute to radiation-induced autophagic cell death in MCF-7 breast cancer cells[29]. However, the potential involvement of P53/DRAM signaling pathway in autophagy induced by antiangiogenic drugs remains unknown. In this study we found that treatment with Conbercept increased the expression of p53 and DRAM. However, P53 inhibitor reversed autophagy-related gene, such as LC3, Beclin-1 and LAMP1.

However, the relationship between P53 and DRAM is not discussed in this study, which will be the focus of the next study.

## Conclusions

Our findings suggest that autophagy is expected to be a new target for improving the effect of neovascularization inhibitors via p53/DRAM signal pathway. However, in spite of the extensive application of RF/6A cells in studies on retinal angiogenesis, it may be different from human choroidal vascular endothelial cells. Therefore, further studies using human choroidal vascular endothelial cells and in vivo models are required to clarify the role of autophagy in angiogenesis.

## List Of Abbreviations

age-related macular degeneration (AMD);proliferative diabetic retinopathy(PDR); central retinal vein occlusion(CRVO);anti-vascular endothelial growth factor (anti-VEGF)

## Declarations

### Ethics approval and consent to participate

This study only involves cell-related experiments and has been approved by the ethics committee of the ninth affiliated hospital of xi 'an jiaotong university.

### Consent for publication

Not applicable

### **Availability of data and materials**

All data generated or analysed during this study are included in this published article.

### **Competing interests**

There are no any ethical/legal conflicts involved in the article. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

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### **Authors' contributions**

YW, study concepts and design,literature research, manuscript preparation,manuscript editing, statistical analysis; YY, study concepts and design, literature research; LR, literature research; BHW, literature research; HQL, data analysis; JC, study concepts and design; ZL, Part of the experimental operation; JHD, guarantor of integrity of the entire study, study concepts and design.

All authors have read and approved the final manuscript.

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Not applicable

### **Disclosure statement**

There are no any ethical/legal conflicts involved in the article. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

### **Data availability statement**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data analyzed during this study are included in this published article.

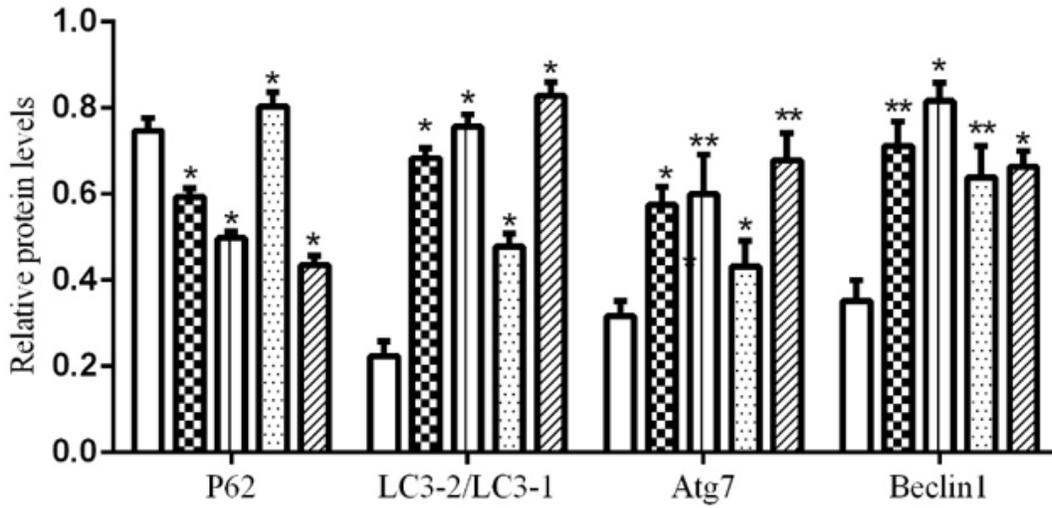
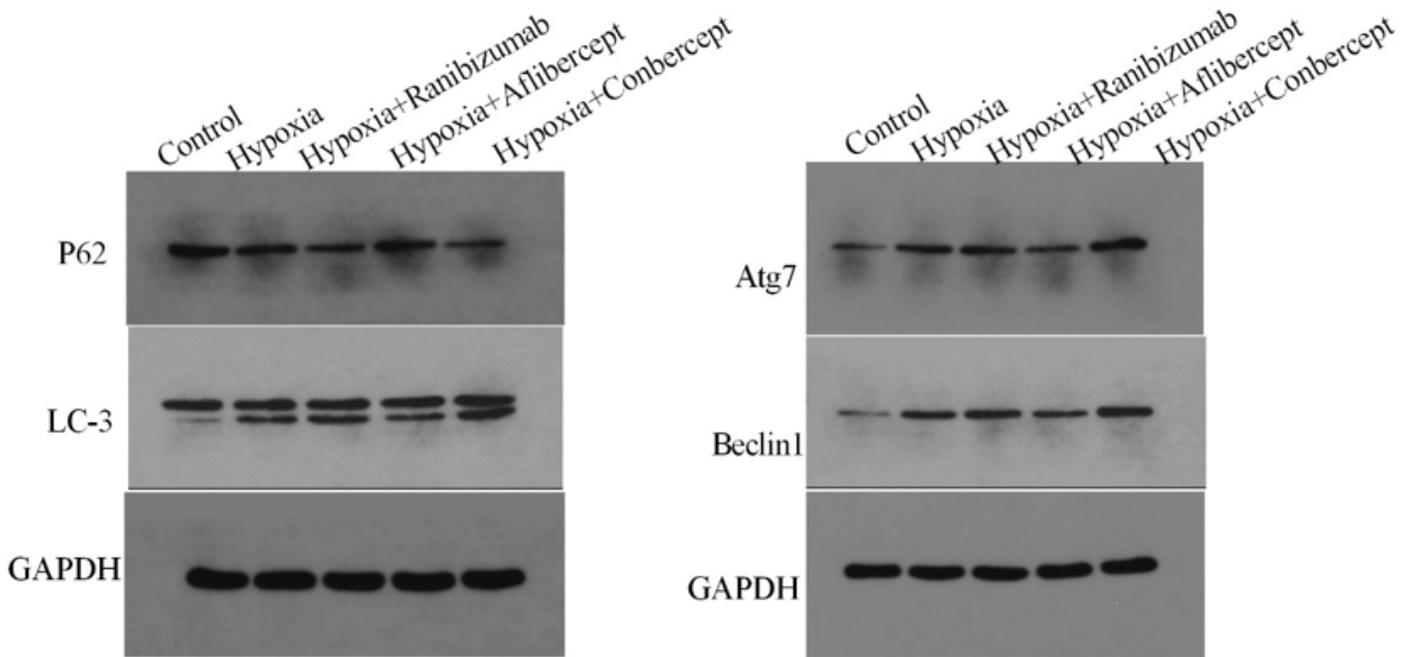
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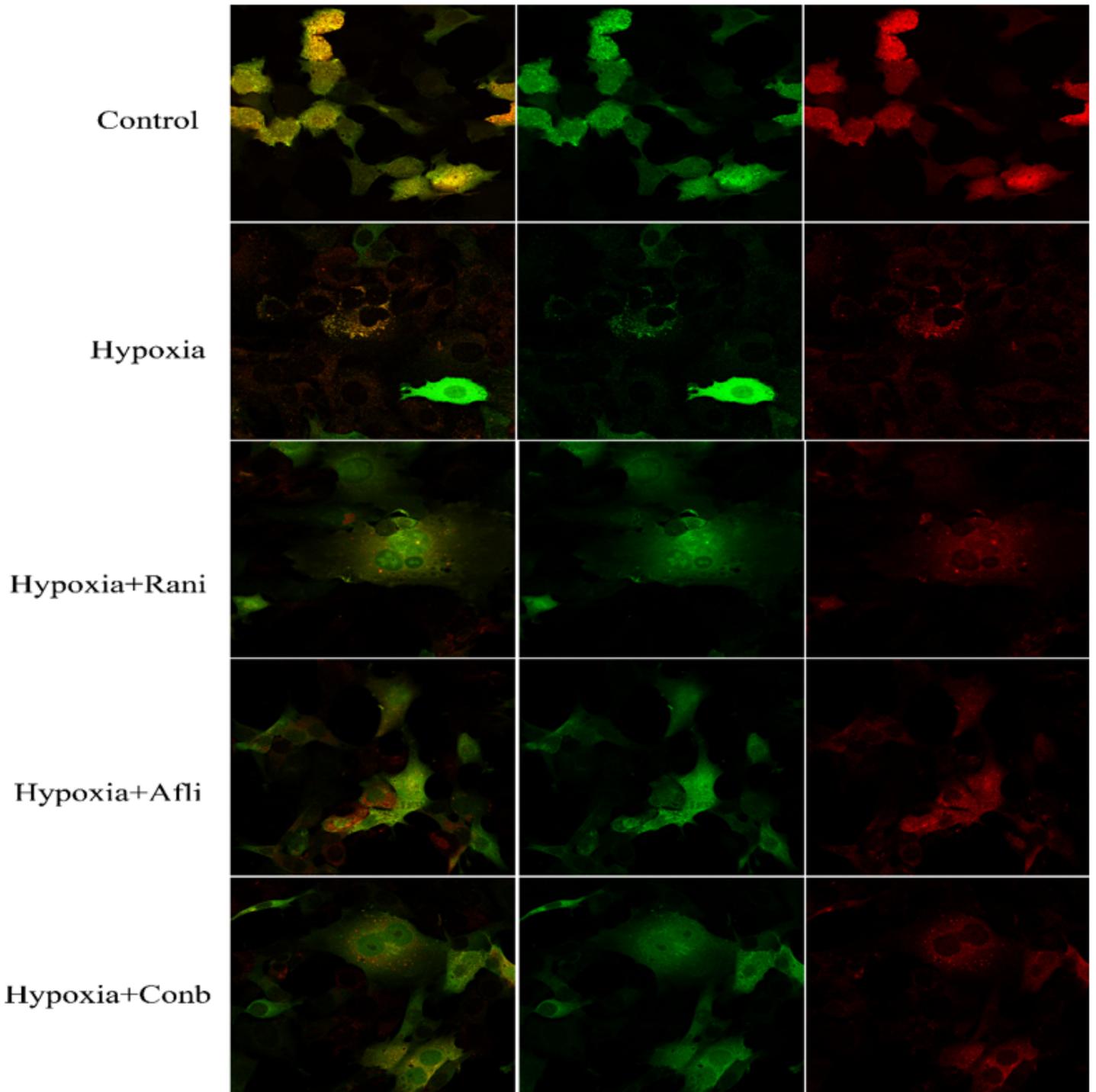
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## Figures



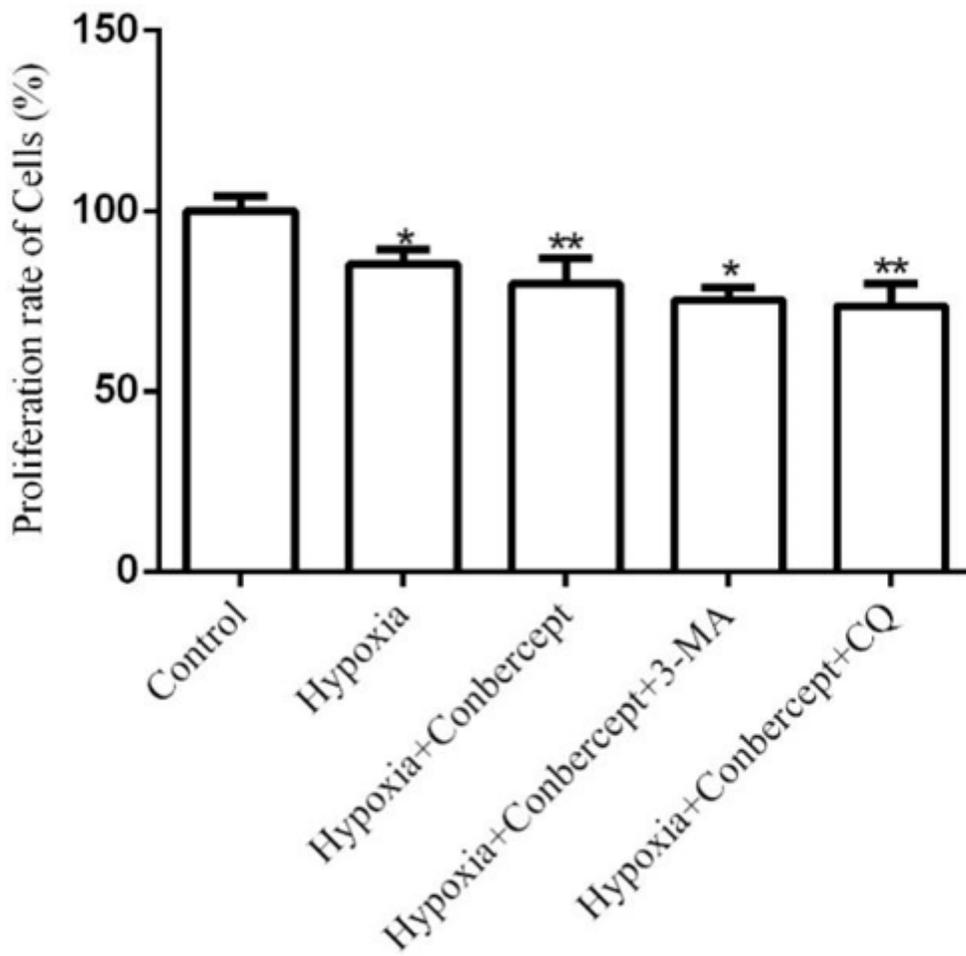
**Figure 1**

The protein level of Beclin1 , LC3-2/1, Atg7 and P62 in Ranibizumab, Conbercept and hypoxia group



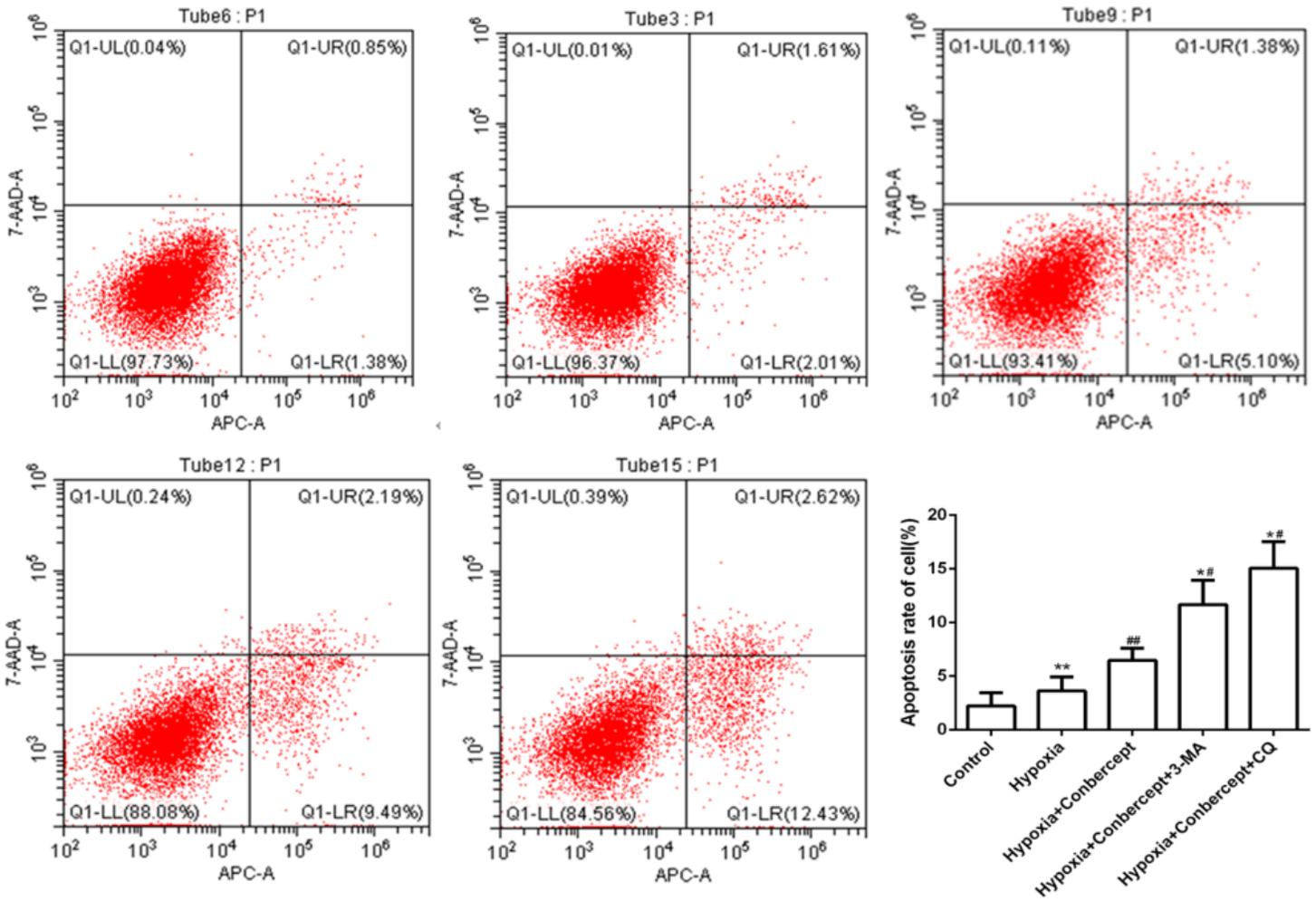
**Figure 2**

The results of autophagic fluxare in Ranibizumab, Conbercept and hypoxia group



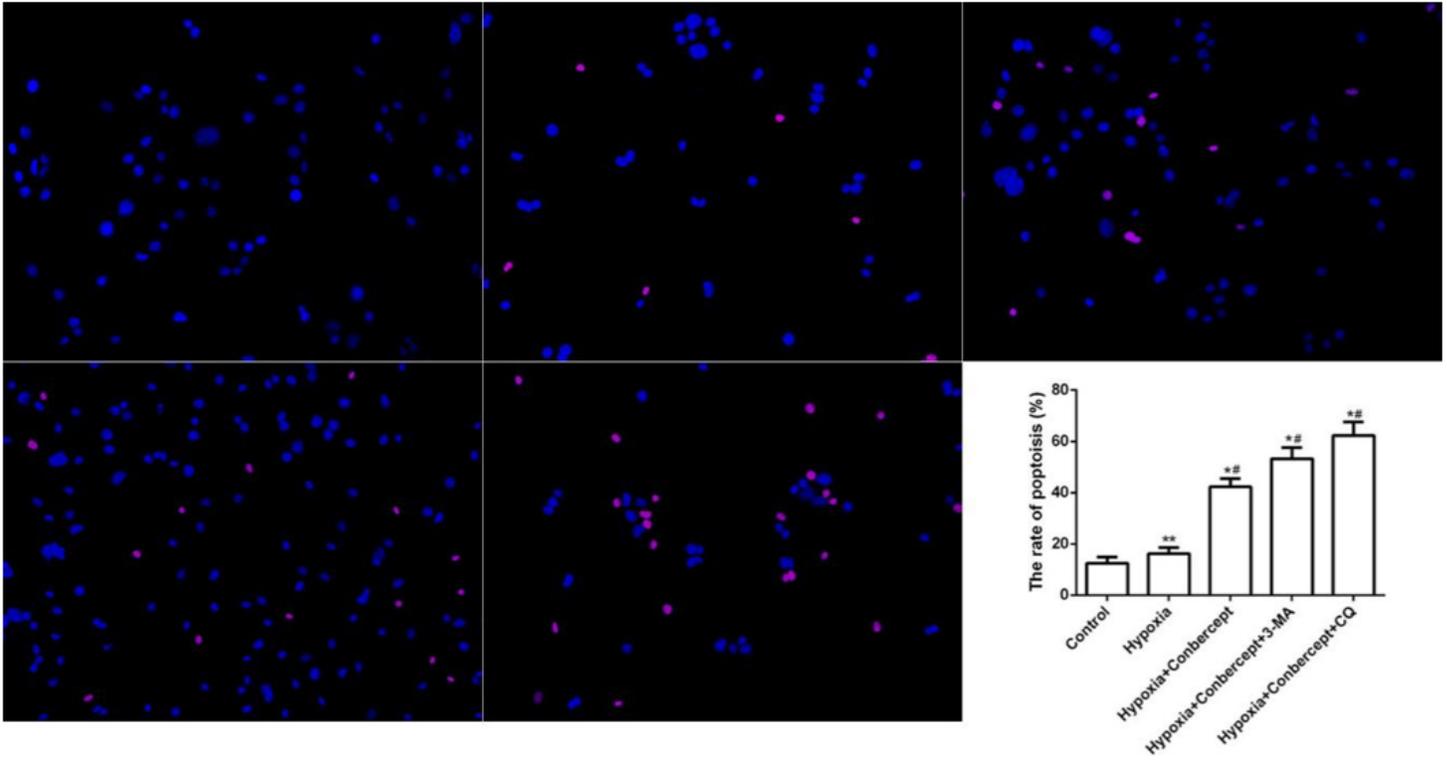
**Figure 3**

The rate of cell proliferation in Ranibizumab, Conbercept and hypoxia groups



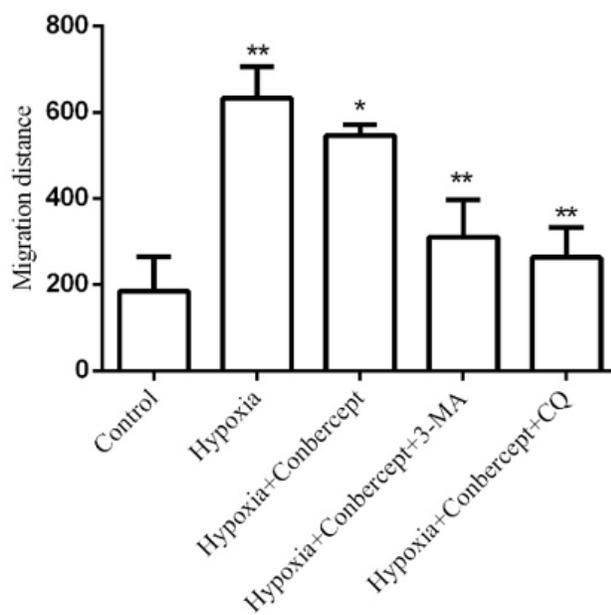
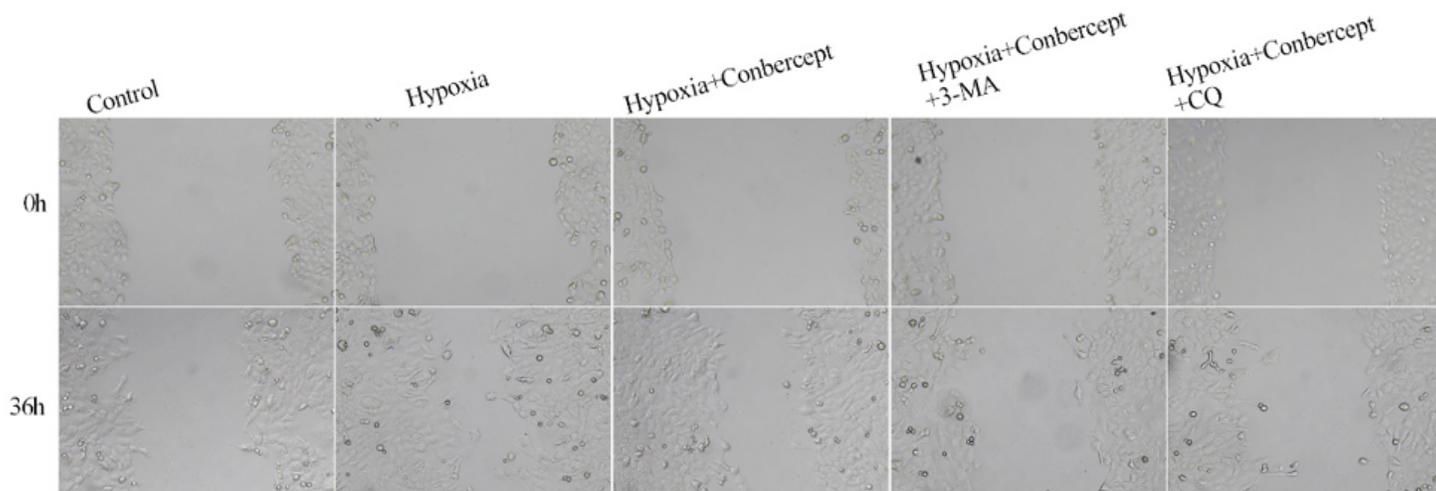
**Figure 4**

The apoptosis rate of RF / 6A cells in Hypoxia+Conbercept, Hypoxia+Conbercept+3-MA, Hypoxia+Conbercept+CQ and hypoxia groups



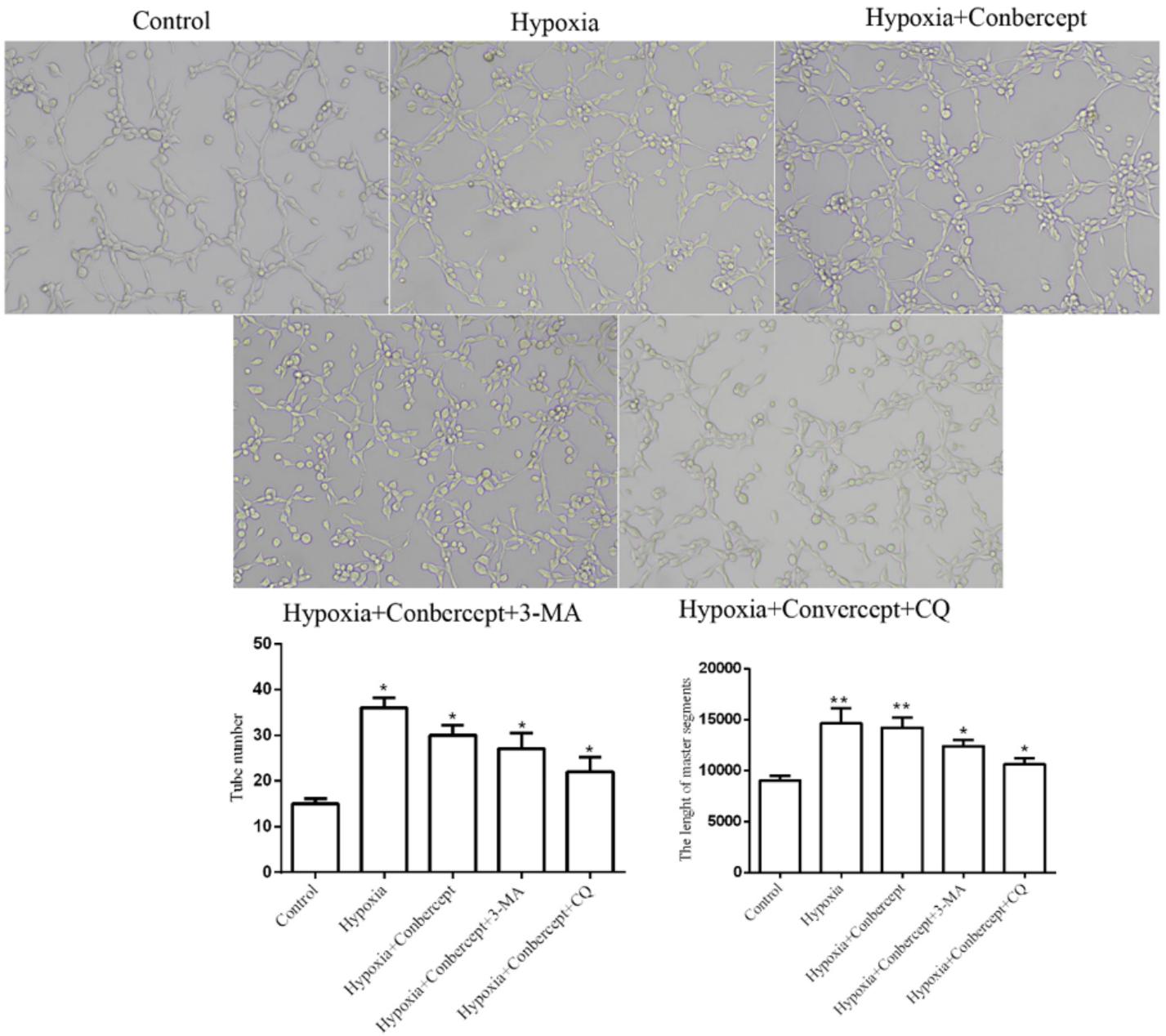
**Figure 5**

The result of TUNEL and flow cytometry in Hypoxia+Conbercept, Hypoxia+Conbercept+3-MA, Hypoxia+Conbercept+CQ and hypoxia groups.



**Figure 6**

The effects of RF/6A cells migration and tube formation Hypoxia+Conbercept, Hypoxia+Conbercept+3-MA, Hypoxia+Conbercept+CQ and hypoxia groups respectively.



**Figure 7**

Master segments length in Conbercept and Conbercept+3-MA group

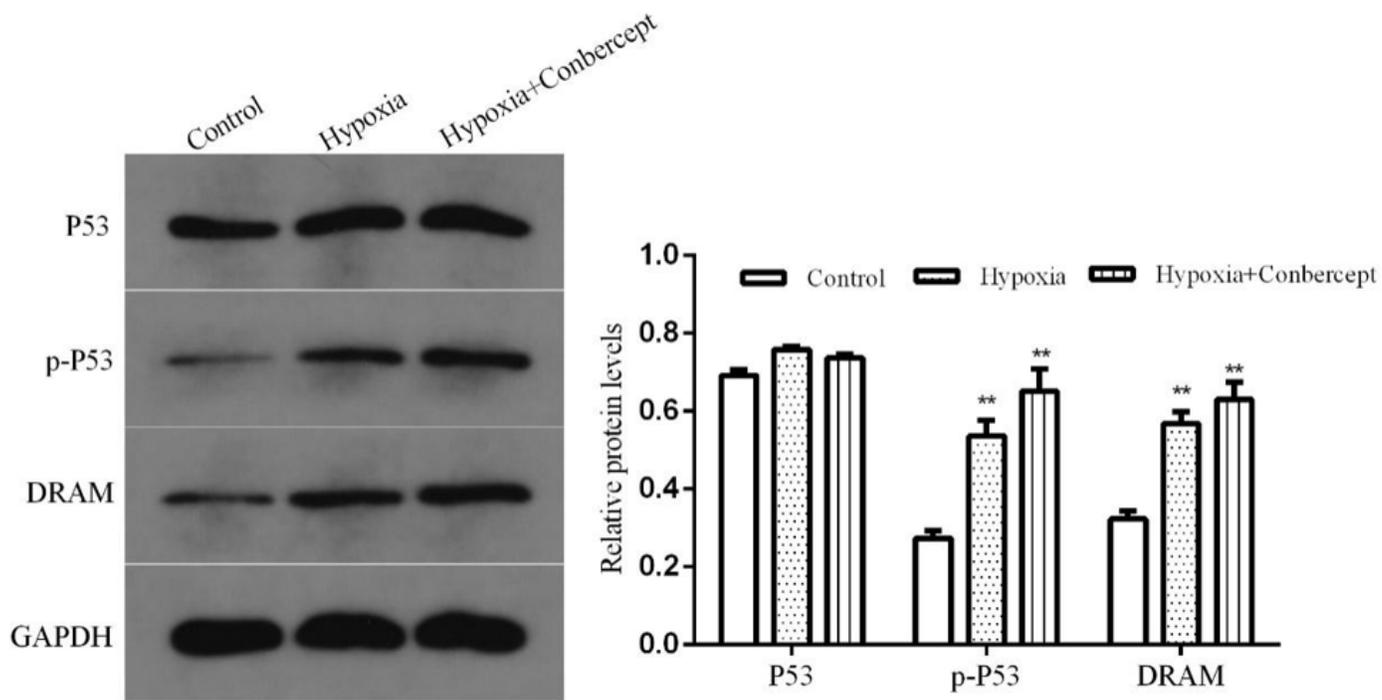


Figure 8

Master segments length in Conbercept and Conbercept+3-MA group

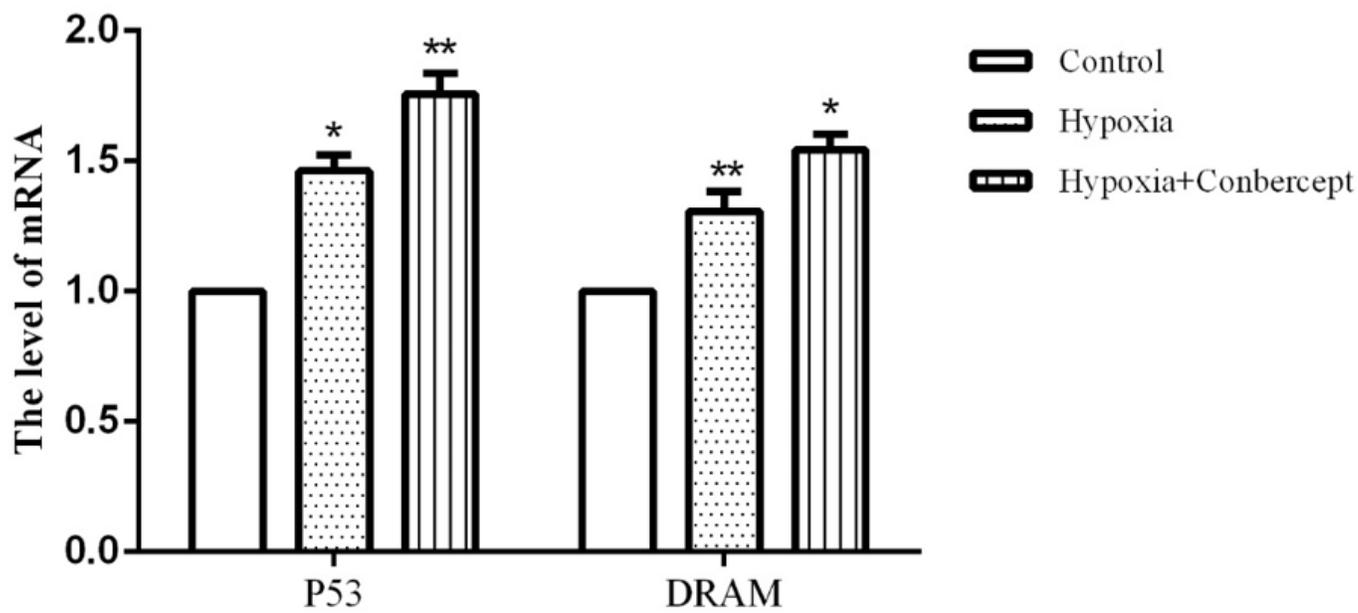
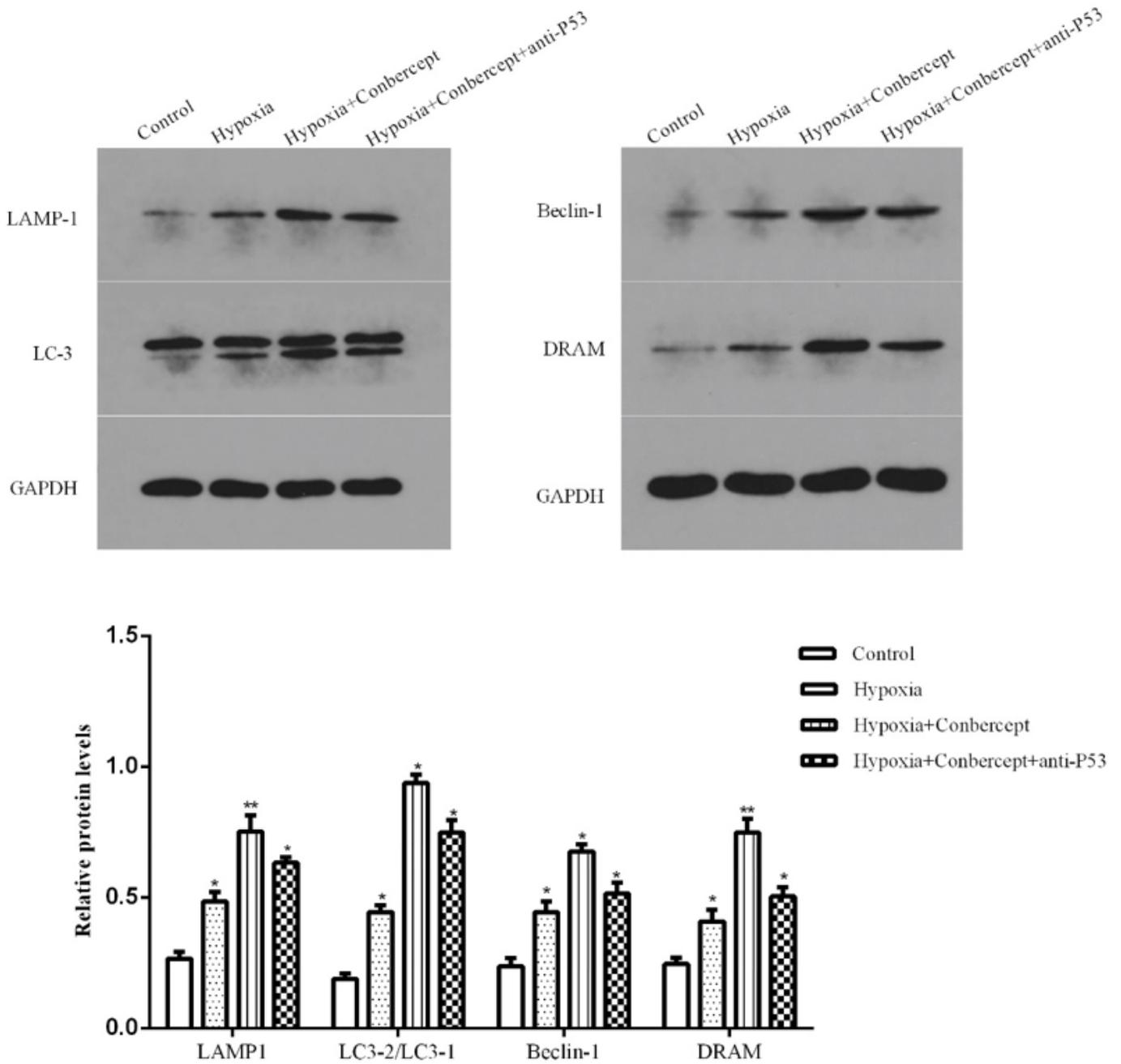


Figure 9

The expression of p53 and DRAM in Hypoxia and Hypoxia+Conbercept by qPCR.



**Figure 10**

The expression of LC-3, LAMP1, Beclin-1 and DRAM in Hypoxia, Hypoxia+Conbercept and Hypoxia+Conbercept+anti-p53 groups.