·Basic Research·

Effects of resveratrol on proliferation of retinal vascular endothelial cells and expression of VEGF

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Abstract

• AIM: To study the effects of resveratrol on the proliferation of human retinal vascular endothelial cells (RVEC) induced by cobalt chloride-simulated hypoxia *in vitro*.

• METHODS: CoCl2(100μmol/L) was used to simulate hypoxic condition, and human RVEC were cultured *in vitro* as model. The cell proliferation was determined by MTT method; SABC method was employed to test the expression of vascular endothelial growth factor (VEGF); and computer image analyzer was used to process data. The effects of resveratrol on the proliferation of vascular endothelial cells were observed.

• RESULTS: Resveratrol inhibited the proliferation of human RVEC induced by $CoCl_2$ in a dose- and time-dependent manner *in vitro*, meanwhile VEGF expression in all groups which were administered medicine was down-regulated. Both kinds of inhibitive effects of resveratrol were statistically significant ($P<0.01$).

• CONCLUSION: Resveratrol can significantly inhibit the proliferation of human RVEC and the expression of VEGF, which may provide a new approach for prevention and treatment of retinal neovascular diseases.

• KEYWORDS: resveratrol; retinal vascular endothelial cells; cell proliferation; vascular endothelial growth factor

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INTRODUCTION

Retinal neovascularization is critical in the occurrence and development of many ocular fundus diseases such as retinopathy of prematurity (ROP), diabetic retinopathy (DR), etc. Retinal neovascularization is formed due to the stimulation of retinal ischemia or hypoxia and leads to serious ocular complications and even blindness. Therefore seeking the effective retinal angiogenic inhibitor is one of difficult problems that clinical ophthalmologists need to tackle currently [1,2]. Resveratrol has multiple roles such as anti-oxidation, anti-proliferation, eliminating free radicals, anti-apoptosis, ameliorating microcirculation and so on [3-10]. The present study established the model of hypoxia-induced retinal vascular endothelial cells (RVEC) cultured *in vitro*, to explore the effects of resveratrol on the proliferation of RVEC and the expression of vascular endothelial growth factor (VEGF), and to provide the experimental evidence for seeking retinal neovascular inhibitors clinically.

MATERIALS AND METHODS

Materials Resveratrol is the standard product provided by National Institute for the Control of Pharmaceutical and Biological Products, the purity of which is over 99%. S-P hypersensitive reagent kit and DAB kit were made by Maixin Company of Fuzhou; DMSO and MTT were from Sigma Corporation; and the automatic enzyme calibration apparatus was of Bio-Rad model 1450, USA.

Methods

Cell culture and appraisal After the informed consents were obtained, donor eyes were taken from those aged 18-45 years old, who underwent corneal transplantation within 12 hours after accidental death. Under sterile conditions, the retinal neuroepithelial layer was peeled off, digested by collagenase and cultivated in the incubator of constant temperature with high sugar DMEM containing 100mL/L fetal bovine serum(FBS), 50mL/L CO_2 at 37℃. The cells had once passage after 2-3 days.

Experiment for the relation between drug concentration and drug effectThe cells were divided into five groups and 100μmol/L cobalt chloride ($CoCl_2$) was used to simulate the hypoxic environment. Among them, four experimental groups were added 10, 50, 100, 200μmol/L resveratrol respectively, and one experimental group was not added resveratrol as control. In addition, a normal blank control group was set up, that is, which is not added $CoCl_2$ or resveratrol. Each well was added 200μL of the above matter, and put into the incubator with 50mL/L CO_2 and

950mL/L air at 37℃ for 24 hours. For every concentration there were three duplicating wells.

Experiment for the relation between time and drug effect The cells were divided into four groups and also 100μmol/L $CoCl_2$ was employed to simulate the hypoxic environment. Among them, three experimental groups were all added 100μmol/L resveratrol, which acted for 12, 24, 36, and 48 hours respectively; the other one experimental group was not added resveratrol as control. Every well was added 200μL of the above matter, and three duplicating wells were established for each concentration.

MTT assay The cells which were cultivated into the fourth generation were digested by 2.5g/L trypsinase, and then 5×10^7/L cell suspension was made through DMEM containing 100mL/L FBS. 200μL of the suspension was inoculated into every well of the 96-well board, which was then placed into the incubator with 50mL/L CO_2 and 950mL/L air at 37℃ for 24 hours of incubation. The cells almost spread fully on the bottom of the well. Then the culture solution was replaced by DMEM which does not contain FBS and incubation was continued for 12 hours. According to the experiment design, $CoCl_2$ and resveratrol or DMEM which does not contain FBS were added, and placed into the incubator with 50mL/L CO_2 and 950mL/L air at 37℃ for 24 hours. After the board was taken out, every well was added 20μL 5g/L MTT solution and culture was continued for 4 hours. When the culture was over, the supernate was abandoned. Every well was added 150μL DMSO to terminate the reaction. Subsequently it was shaken for 10 minutes , and immediately put on the instrument to perform enzyme-linked immunosorbent assay (ELISA) to determine the absorbance value of each well. The wavelength was 490nm. The inhibitive rate of the drug on cell proliferation was calculated.

Immunohistochemical staining The cells were divided into 5 groups and 100μmol/L cobalt chloride ($CoCl_2$) was used to simulate the hypoxic environment, in which four experimental groups were added 10, 50, 100, 200μmol/L resveratrol respectively and one experimental group was not added resveratrol as control. Besides, a normal blank control group was set up in which neither $CoCl_2$ nor resveratrol was added. Every well was added 200μL of the above matter, and then the board was put into the incubator with 50mL/L CO_2 and 950mL/L air at 37℃ for 24 hours of incubation. For each concentration there were three duplicating wells.

With regard to the preparation of cell growing on the glass, the RVEC of the second to fourth generation cultured on single layer were digested and fell off the wall. At the cell density of 5×10^7/L, 1mL cell solution was inoculated into a 24-well board on which there was coverglass with the size of 4mm ×4mm placed in advance. After 24 hours, the bottom of the well was nearly covered with cells. Then the DMEM that does not contain FBS substituted for the culture solution and the incubation was kept on for 12 hours. In the light of the above experiment design, resveratrol, $CoCl_2$ or DMEM that does not contain FBS were added, and the culture solution was put into the incubator with 50mL/L CO_2 and 950mL/L air at 37℃ . After 24 hours of incubation the coverglass was collected.

With respect to the detection and quantification of VEGF, the coverglass was washed with 0.1mol/L phosphate buffer solution (PBS) three times, 3 minutes per time; fixed in 40g/L paraformaldehyde at room temperature for half an hour; and washed thoroughly with distilled water. After fixation the coverglass was put into the peroxidase block solution (reagent A) at room temperature to incubate for 10 minutes, and the endogenous peroxidase was inactivated; washing with 0.1mol/L PBS three times, 3 minutes per time; the normal non-immune animal serum (reagent B) was added to incubate at room temperature for 10 minutes; the first antibody (mouse anti-human VEGF monoclonal antibody) was added and placed into the refrigerator at 4℃ overnight; washing with 0.1mol/L PBS three times, 3 minutes per time; the second biotin-labeled antibody (goat anti-mouse IgG, reagent C) was added to incubate at room temperature for 10 minutes; washing with 0.1mol/L PBS three times, 3 minutes per time; the streptavidin peroxidase (SP, reagent D) solution was added to incubate at room temperature for 10 minutes; washing with 0.1mol/L PBS three times, 3 minutes per time; then DAB was used to show color and it took about 3 minutes to show color; washing with running water; the hematoxylin was applied for counter staining for 1-2 minutes; dehydrated and mounted. The results of staining were analyzed by the image analyzer. The concrete method was as follows: four slices were taken from each group, and 15 cells were selected from each slice to measure the absorbency in the cytoplasm. In the control group, PBS was used to substitute for the first antibody as negative control; the remained steps were the same.

RESULTS

Effect of Resveratrol on the Proliferation of RVEC

Dose-effect relationship Compared with the blank control group, $CoCl_2$ could promote the proliferation of RVEC remarkably ($P<0.01$). Compared with $CoCl_2$ group, except the group of 10μmol/L resveratrol, in the other three groups of 50, 100, 200μmol/L resveratrol, resveratrol inhibited the

proliferation of RVEC induced by $CoCl_2$ to different extent. There were statistically significant differences (P<0.01), and the inhibitive rates were 14.99%, 36.07% and 43.91% respectively. What's more, there existed significant differences among four resveratrol groups of different concentrations (P<0.01) (Table 1).

Time-effect relationship In comparison with $CoCl_2$ group, when 100μmol/L resveratrol acted on human RVEC induced by $CoCl_2$ for 12, 24, 36, 48 hours, the inhibitive rates were 3.56%, 35.63 %, 51.74%, 63.58% respectively. The inhibitive effect was increased with time prolongation. As 100μmol/L resveratrol acted on human RVEC for 12-48 hours, there were significant differences in its inhibitive effect (P<0.01) compared with the condition at 12 hours (Table 2).

Effect of Resveratrol on the Expression of VEGF The absorbency measured by the image analyzer was carried out statistical analysis. It was found that in the three groups of 50, 100, 200μmol/L resveratrol, resveratrol could inhibit the expression of VEGF of retinal vascular endothelial cells in-duced by $CoCl_2$ significantly (P<0.01) except the group of 10μmol/L resveratrol. Its inhibitive rates were 27.05%, 37.15% , 50.97% respectively. Moreover, there existed significant differences among four resveratrol groups of diffe- rent concentrations (P<0.01) (Table 3).

Table 1 Effects of resveratrol on the proliferation of RVEC induced by $CoCl_2$ $\bar{x}\pm s$

Group	Concentration of Resveratrol(μmol/L)	A value
Blank control group		0.552±0.006
$CoCl_2$ group		0.854±0.004
$CoCl_2$+ resveratrol group 1	10	0.854±0.006
$CoCl_2$+ resveratrol group 2	50	0.726±0.006[b]
$CoCl_2$+ resveratrol group 3	100	0.546±0.009[b]
$CoCl_2$+ resveratrol group 4	200	0.479±0.007[b]

[b]P<0.01 *vs* $CoCl_2$ group

Table 2 Effects of 100μmol/L resveratrol on RVEC proliferation induced by $CoCl_2$ for different time $\bar{x}\pm s$

Group	Resveratrol group	$CoCl_2$ group
12h group	0.786±0.009	0.815±0.007
24h group	0.569±0.006[bd]	0.884±0.007
36h group	0.471±0.006[bd]	0.976±0.016
48h group	0.382±0.005[bd]	1.049±0.005[b]

[b]P<0.01 *vs* 12h group; [d]P<0.01 *vs* $CoCl_2$ group

Table 3 Effects of resveratrol on the expression of VEGF of RVEC induced by $CoCl_2$ $\bar{x}\pm s$

Group	Concentration of Resveratrol(μmol/L)	A value
Blank control group		93.39±0.48
$CoCl_2$ group		334.66±1.23
$CoCl_2$+ resveratrol group 1	10	333.99±0.46
$CoCl_2$+ resveratrol group 2	50	244.14±0.61[b]
$CoCl_2$+ resveratrol group 3	100	210.33±0.79[b]
$CoCl_2$+ resveratrol group 4	200	164.10±0.77[b]

[b]P<0.01 *vs* $CoCl_2$ group

DISCUSSION

At present, a great deal of research has been made home and abroad in terms of the mechanism of neovascularization.

Neovascularization is a dynamic process of continuous integration involving the participation of manifold factors. First, the external basement membrane of capillaries undergoes degradation, and vascular endothelial cells migrate to extracellular matrix under the stimulation of factors that promote angiogenesis; secondly, the proliferation of endothelial cells occurs and tubular structures form; finally, new blood vessels differentiate and link to form a new vascular network. It has been proved that factors which promote angiogenesis and endothelial cells play crucial roles in the neovascularization [11]. One of the most important cytokines which promote angiogenesis is VEGF[12-15], which is a neovascular factor arousing great concern in recent years. VEGF can strongly boost the proliferation of endothelial cells, increase the synthesis of extracellular matrix, and facilitate the angiogenesis. The receptor of VEGF, that is, VEGFR, has strong affinity and may specifically bind to VEGF. Some researches have demonstrated that the biological effect of VEGF is fulfilled through two receptors tyrosine kinase (FLt1, KDR) on the surface of endothelial cells. VEGF plays a role of central regulation in the process of vascular growth and is the most important and necessary substance for the neovascularization. The ultimate purpose of investigating the mechanism of neovascuarization is to seek medicine that could inhibit the retinal neovascularization, so that the development of proliferative vitreoretinopathy (PVR) can be restrained such as retinopathy of prematurity, diabetic retinopathy, etc. So far there is not effective medicine to control retinal neovascularization. Resveratrol belongs to polyphenol compounds, mainly existing in grape, veratrum, polygonum cuspidatum and other plants. Its chemical name is Trans-3-4'-trihydroxystilbene. Now it has been shown that resveratrol possesses multiple biological functions such as anti-platelet aggregation, anti-inflammation, eliminating free radicals, anti-tumor, antioxidation, anti-apoptosis, protecting cardiovascular system and so on [3,4]. Some studies have indicated that resveratrol could attenuate the activation of NF-kappaB signal transduction passway caused by oxidized very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in PC12 cells, which resulted in the prevention of apoptosis [5]. Burkitt *et al* [6] verified that the anti-oxidating function of resveratrol was stronger than vitamin E; resveratrol was able to eliminate free radicals

especially hydroxyl free radical and protect DNA from damage. Furthermore, resveratrol can make glutathione in a reduced state through inhibiting GSSG formation, which leads to the suppression of hydroxyl free radical [7]. Resveratrol is a strong capture agent of superoxide anion free radical and hydroxyl free radical, meanwhile it is capable of inhibiting the activities of AP-1, NF-κB and relevant kinases such as MEK, JNK through impeding the production of ROI and blocking lipid peroxidation, which brings about the prevention of cell proliferation. Besides, some research showed that resveratrol has strong ability to inhibit the synthesis of DNA to murine mastocytoma cell strain p815 and human myelocytic leukemia cell strain K562. Its potential mechanism is that resveratrol has tyrosyl which may clear out RNA reductase of small proteins to restrain the activity of RNA reductase [16]. Moreover, resveratrol can suppress DNA polymerase, which not only reduces the ability of DNA synthesis basically to achieve the goal of hindering cell proliferation [17], but also induces the activation of cysteine protease caspase-3 to decompose DNA repair enzyme (poly [ADP-ribose] polymerase)[18]. All these studies suggested that resveratrol can inhibit the cell proliferation but the mechanism is very complicated.

MTT colorimetric assay is a simple, convenient, hypersensitive method to detect mitochondrial dehydrogenase in living cells. The succinate dehydrogenase in living cell mitochondria could make ectogenous yellowish MTT reduced to insoluble purple blue crystal formazan which becomes sediments in cells. Furthermore, there is linear relationship between the number of surviving cells and A value of MTT crystals, whereas the dead cells do not have such a function. Therefore, in a certain range of cell quantity, the level of the content of crystals can reflect the cell activity and indirectly reflect the condition of cell proliferation. The present study probed the effect of resveratrol on the proliferation of RVEC induced by hypoxia. With the increase of drug concentration and prolongation of acting time, its inhibitive effect was enhanced. Moreover, resveratrol has significantly inhibitive effects on the expression of VEGF of human retinal vascular endothelial cells induced by $CoCl_2$ in a dose-dependent manner.

In conclusion, the results of our study showed that resveratrol could markedly suppress the proliferation of retinal vascular endothelial cells and the expression of VEGF, which suggests that resveratrol has the potential application prospects for the treatment of retinal neovascularized diseases. This also provides a new way of thinking for medication of retinal neovascularized diseases. However, the exact action mechanism of resveratrol is not clear yet, and further studies remain to be done.

REFERENCES

1 Song H, Ren B, Gao XW. Advance in medicine therapy for retinal neovascularization. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2008;8(2):358-360

2 Li Z, Ni WJ. Advances of biological drugs for retinal neovascularization. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2007;7(4):1119-1123

3 Roemer K, Mahyar-Roemer M. The basis for the chemopreventive action of resveratrol. *Drugs Today(Barc)* 2002;38(8):571-580

4 Dong Z. Molecular mechanism of the chemopreventive effect of resveratrol. *Mutat Res* 2003;523-524:145-150

5 Draczynska-Lusiak B, Chen YM, Sun AY. Oxidized lipoproteins activate NF-kappaB binding activity and apoptosis in PC12 cells. *Neuroreport* 1998;9(3):527-532

6 Burkitt MJ, Duncan J. Effects of trans-resveratrol on copper-dependent hydroxyl-radical formation and DNA damage: evidence for hydroxyl-radical scavenging and a novel, glutathione-sparing mechanism of action. *Arch Biochem Biophys* 2000;381(2):253-263

7 Hung LM, Chen JK, Huang SS, Lee RS, Su MJ. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. *Cardiovasc Res* 2000;47:549-555

8 Reiter E, Azzi A, Zingg JM. Enhanced anti-proliferative effects of combinatorial treatment of delta-tocopherol and resveratrol in human HMC-1 cells. *Biofactors* 2007;30(2):67-77

9 Yang Y, Paik JH, Cho D, Cho JA, Kim CW. Resveratrol induces the suppression of tumor-derived CD4+CD25+ regulatory T cells. *Int Immunopharmacol* 2008;8(4):542-547

10 Sun W, Wang W, Kim J, Keng P, Yang S, Zhang H, Liu C, Okunieff P, Zhang L. Anti-cancer effect of resveratrol is associated with induction of apoptosis via a mitochondrial pathway alignment. *Adv Exp Med Biol* 2008;614:179-186

11 Du GJ, Wang LL, Wang MW, Li S. Bcl-2 protein, a new anti-angiogenesis target. *Chin J Pharmacol Toxicol* 2004;18(4):317-320

12 Nishimoto-Hazuku A, Hirase T, Ide N, Ikeda Y, Node K. Simvastatin stimulates vascular endothelial growth factor production by hypoxia-inducible factor-1alpha upregulation in endothelial cells. *J Cardiovasc Pharmacol* 2008;51(3):267-273

13 Hirao T, Saga T, Kusukawa J, Yamaki K. Angiogenesis and developmental expression of vascular endothelial growth factor in rat lingual papillae. *Kurume Med J* 2007;54(1-2):9-24

14 Chen D,Fan YC,Li LY.Progress of researches on anti-VEGF medication for neovascular oculopathy. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2008;8(1):123-125

15 Liu WH, Li YZ, Wu YQ. Vascular endothelial growth factor and intraocular neovascularization. *Int J Ophthalmol(Guoji Yanke Zazhi)* 2007;7(6):1681-1684

16 Fontecave M,Lepoivre M,Elleingand E,Gerez C,Guittet O.Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *FEBS Lett* 1998;421(3):277-279

17 Sun NJ,Woo SH,Cassady JM,Snapka RM.DNA polymerase and topoisomerase II inhibitors from Psoralea corylifolia. *J Nat Prod* 1998;6l(3):362-366

18 Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Aggarwal BB. Resveratrol blocks interleukin-1beta-induced activation of the nuclear transcription factor NF-kappaB, inhibits proliferation, causes S-phase arrest, and induces apoptosis of acute myeloid leukemia cells. *Blood* 2003;102(3):987-995