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Simvastatin inhibits HIF-1α and VEGF expression in RPE cells under hypoxia conditions

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辛伐他汀抑制缺氧条件下人视网膜色素上皮细胞 HIF-1α 和 VEGF 的表达水平

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摘要

目的:探讨缺氧条件下辛伐他汀(Sim)对体外培养的人视网膜色素上皮(RPE-19)细胞的影响及其可能机制。

方法:将体外培养的人 RPE-19 细胞随机分为三组:对照组、缺氧组(培养基中 $CoCl_2$ 的最终浓度为 125μmol/L)和 Sim 处理组(在含有 125μmol/L $CoCl_2$ 的 RPE-19 细胞培养基中加入 3μmol/L Sim)。24h 后,观察 RPE-19 细胞的形态,用 MTT 法检测细胞增殖,采用酶联免疫吸附试验(ELISA)和免疫印迹法检测缺氧诱导因子 1-α(HIF-1α)和血管内皮生长因子(VEGF)的分泌水平和蛋白表达,采用免疫印迹法检测自噬蛋白的表达水平,TUNEL 法检测细胞凋亡。

结果:缺氧条件下 RPE-19 细胞的形态发生了明显变化。缺氧组 RPE-19 细胞中 HIF-1α 和 VEGF 的蛋白表达明显增加,Sim 治疗后显著降低。Beclin1 和 LC3B 蛋白在 $CoCl_2$+Sim 组中的表达水平有所下降,且表达水平显著低于对照组和 $CoCl_2$ 组。缺氧条件下,Sim 抑制了 RPE-19 细胞增殖,促进了细胞的凋亡。

结论:Sim 可抑制缺氧条件下 RPE-19 细胞 HIF-1α 和 VEGF 蛋白的表达,抑制细胞增殖,促进凋亡,Sim 促进 RPE-19 细胞凋亡的机制可能与其抑制自噬有关。

关键词:视网膜色素上皮细胞;缺氧;辛伐他汀;脉络膜新生血管

Abstract

• AIM: To investigate the effects of simvastatin (Sim) on human retinal pigment epithelial cells (RPE-19) and the possible mechanisms *in vitro* under hypoxia.

• METHODS: RPE-19 cells were divided into three group: control group, hypoxia group (the final concentration of $CoCl_2$ in the medium was 125 μmol/L), and Sim treatment group (3 μmol/L Sim was added in the RPE-19 cells' medium which contain 125 μmol/L $CoCl_2$). After 24h, the morphology of RPE-19 cells were observed, the proliferation of cells were calculated by MTT, the secretion levels and protein expression of hypoxia-inducible factor 1-Alpha (HIF-1α) and vascular endothelial growth factor (VEGF) were detected by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The expression level of autophagy protein was detected by Western blot and apoptosis was detected by TUNEL.

• RESULTS: The morphology and activity of RPE-19 cells showed an apparent change under hypoxia. The expression of HIF-1α and VEGF protein were increased obviously in the hypoxia group and then significantly decreased after Sim treatment. Beclin1, and LC3B proteins

were decreased in the $CoCl_2$ + Sim group, and the expression levels were lower than the control and $CoCl_2$ group. Under hypoxia, Sim inhibited RPE-19 cells' proliferation and promoted the apoptosis.

• CONCLUSION: Sim inhibits RPE-19 cells' proliferation, decreases HIF-1α and VEGF protein, and promotes apoptosis under hypoxia. Our results suggested that the mechanism by which Sim promoted apoptosis in RPE-19 cells may be related to its inhibition of autophagy.

• KEYWORDS: retinal pigment epithelial (RPE) cells; hypoxia; Simvastatin; choroidal neovascularization
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INTRODUCTION

Age-related macular degeneration (ARMD) is the highest blinding disease in developed countries[1]. 85% of patients present a dry ARMD, while the wet (new blood vessel) form affects the remaining 15% and usually develops from the dry form of ARMD. Retinal pigmented epithelial (RPE) cells located between the neuroretina and choroid are monolayer pigmented epithelial cells. It is known that RPE dysfunction plays an significant role in the pathogenesis of ARMD[2-4]. Statins or HMG CoA (3-hydroxy-3-methylglutaric coenzyme A) reductase inhibitors blocking cholesterol biosynthesis and up-regulating LDL receptor expression through methylovalerate pathways has been widely used to reduce serum cholesterol levels. Since cardiovascular risk factors are also related to ARMD, interventions to reduce cardiovascular risk factors, such as statins, maybe also contribute to the treatment of ARMD. Many studies have focused on the relationship between statins and ARMD, but the conclusion remains controversial[5-8]. Therefore, more evidence is needed to verify the effect of statins on ARMD. It is known that VEGF is a key cytokine to promote angiogenesis[9]. It promotes the division and proliferation of endothelial cells and increases vascular permeability. Clinical studies have found that VEGF can effectively inhibit choroidal neovascularization (CNV). Increasing evidence has shown that hypoxia contributes to the development of CNV, and RPE cells played a causative role in VEGF production during the formation of CNV[10]. It has been reported that simvastatin (Sim) up-regulates the expression of VEGF in vascular endothelial cells through the HIF-1α signaling pathway and promotes neovascularization[11]. While another study showed that atorvastatin inhibits VEGF expression and finally inhibits the CNV development in mice[12]. Thus we conducted this study to confirm the effects of statins on HIF-1α and VEGF in RPE cells and tried to reveal the possible mechanisms.

MATERIALS AND METHODS

Cell Culture RPE-19 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C, indoor air humidity of 95% and carbon dioxide concentration of 5%. The medium was changed after 24h of culture. RPE-19 cells were randomly divided into different groups: control group and hypoxia group (the final concentration of $CoCl_2$ in the cultured medium was 125 μmol/L), and Sim group (the final concentration of Sim in the cultured medium was 3 μmol/L). At the time of 24h, group of cells were observed and photographed with the inverted microscope. Western blotting and Enzyme-linked immunosorbent assay (purchased from Ex-Cell Biology Inc. Shanghai, China, EH015-96) were used to detect HIF-1α and VEGF protein expression (purchased from BD Biosciences, cat:555036) and secretion level.

MTT Cell Proliferation Assay The logarithmic growth phase RPE-19 cells were digested and added to the cell culture mediums. Cells were then adjusted to a suspension with 5×10^4 cells/mL and inoculated on 96 well plate (100 μL per well). According to group design, relevant factors were added and seven replicate wells were designed for each group. The culture plate was transferred to a CO_2 incubator for cultivation. After 24h, 10 μL MTT solution was added to each well and incubated for 4h. After centrifugation, remove the supernatant, remove 100 μL of solution and shaked for 10min. The absorbance (A) value was measured at 490 nm with an automatic microplate spectrophotometer. The experiment was repeated three times ($n=3$).

Western Blotting RPE-19 cells were collected and treated to detect HIF-1α and VEGF. After treatment, the separated proteins were transferred to nitrocellulose membranes and then blocked with Tris-buffered saline (TBS)-T buffer containing 5% non fat emulsion β-actin (1∶500, Santa Cruz Biotech, CA, USA), HIF-1α (1∶500, Santa Cruz Biotech, CA, USA), VEGF (1∶500, Santa Cruz Biotech, CA, USA), LC3B (1∶500, Santa Cruz Biotech, CA, USA), Beclin-1 (1∶500, Bioword technology, MN, USA), p62 (1∶500, Santa Cruz Biotech, CA, USA), and then incubated with horseradish peroxidase (HRP)-bound secondary antibody at room temperature for 1h. The labeled bands were visualized and quantified using a chemiluminescence imaging system (CliNX, Shanghai, China). Use CliNX analysis software to scan and count the gray value. The ratio of target protein gray value/actin was expressed as the relative expression level of target protein.

Enzyme-linked Immunosorbent Assay Add sample diluent 100 μL to blank well, standard or test sample 100 μL to the rest well. The plate was coated and incubated at 37℃ for 90min. Dispose the liquid, add chromogen solutions A and B 100 μL to each well (prepared within 15min before use), add film covering to the HR-labeled plate, incubate at 37℃ for 1h. Wash the plate 3 times, add HRP conjugate working solution (prepared within 15min before use) 100 μL to each well, add film mulching, incubate at 37℃ for 30min. Discard the liquid in the hole, shake dry and wash the plate for 5 times, using the same method as Step 1. Add substrate solution 90 μL to each well , and incubate with HR-labeled

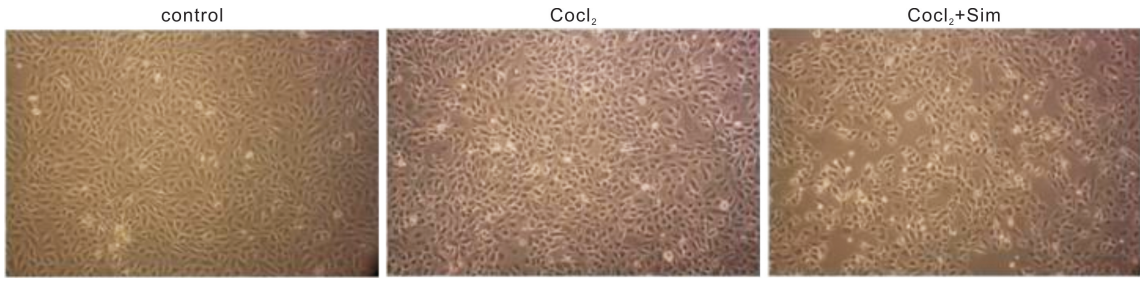

Figure 1 After 12h of cell culture, the morphology of RPE-19 cells in $CoCl_2$ and $CoCl_2$+Sim group was observed under hypoxia Sim is short of simvastatin.

plate coated with film at 37℃ for 15min. Add Stop Solution 50 μL to each well, stop the reaction, the blue color turns yellow. Immediately measure the optical density (OD value) of each hole at 450 nm with a microplate analyzer.

TUNEL Assay Apoptosis was analyzed using the One Step TUNEL Apoptosis Assay kit (C1088, Beyotime Institute of Biotechnology), according to the manufacturer's instructions. RPE-19 cells were placed on a glass cover and fixed with 4% paraformaldehyde and 70% ethanol for 30min at room temperature. The slides were permeated with Triton X-100 at room temperature for 5min and washed with PBS for 3 times. The slides were then sealed in 3% H_2O_2 for 10min at room temperature and washed 3 times with PBS. Add 50 μL TDT enzyme reaction solution, keep it at 37℃ for 60min, and wash it with PBS solution for 5min, three times. Add 50 μL streptomycin horseradish peroxidase solution, keep it at 37℃ for 30min, and wash it with PBS solution for 5min, three times. 50 μL DAB working solution was dripped and kept at room temperature for 10min. The number of apoptotic cells was obtained. It is used to reflect the severity of apoptosis in RPE-19 cells.

Statistical Analysis Statistical analyses were used Statistical Products and Services Solutions (SPSS) 26.0 software program. All quantitative data were represented at least three independent experiments. The data were expressed as the Mean of the Standard Deviation. The differences between groups were tested by analysis of variance (ANOVA). The paired LSD-T test was used for comparison among groups. The two tailed P-value with $P<0.05$ was considered significant.

RESULTS

RPE-19 Cells' Morphology Observed Under Inverted Phase Contrast Microscope After cultured for 12h, the morphology of RPE-19 cells in each group was observed. RPE-19 cells in the control group were typical polygonal. Irregular round cells increased in hypoxia group. After treated with Sim under hypoxia, cells number were decreased compared with the hypoxia group (Figure 1).

Sim Inhibits RPE-19 Cells Proliferation Under Hypoxia Conditions The effect of Sim on the RPE-19 cells' proliferation was investigated by MTT assay (Figure 2). Our results showed that cell proliferation was significantly increased under hypoxia ($P<0.05$). When exposed to Sim under hypoxia, RPE-19 cell proliferation was reduced obviously.

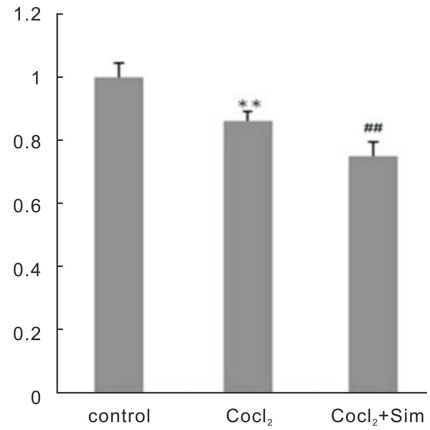

Figure 2 Effects of Sim on the proliferation of RPE-19 cells $^{**}P<0.05$ *vs* control; $^{\#\#}P<0.05$ *vs* $CoCl_2$ group. Sim is short of simvastatin.

Table 1 The levels of HIF-1α and VEGF were determined by ELISA (24h)

Groups	HIF-1α (pg/mL)	VEGF (pg/mL)
Control	29.06±0.96	59.62±0.77
$CoCl_2$	106.56±0.48[a]	180.51±1.02[a]
$CoCl_2$+Sim	80.17±0.83[b]	126.51±0.77[b]
F	35.26	33.28
P	0.001	0.001

VEGF: Vascular endothelial growth factor; [a]$P<0.05$ *vs* control; [b]$P<0.05$ *vs* $CoCl_2$ group. Sim is short for simvastatin.

Sim Inhibited the Protein Expression of HIF-1α, VEGF, and LC3B Under Hypoxia To study the effect of statins on HIF-1α and VEGF in RPE cells under hypoxia conditions, we detected HIF-1α and VEGF by western blotting, we also detected the expression of autophagy-related protein LC3B (Figure 3). Our results showed that when RPE-19 cells were treated with $CoCl_2$, the level of HIF-1α, VEGF, and LC3B protein expression were increased and then decreased after Sim treatment.

Effects of Sim on the Secretion Levels of HIF-1α and VEGF in RPE Cells Under Hypoxia ELISA results showed that hypoxia up-regulated the secretion levels of HIF-1α and VEGF expression. And they reduced when treated with Sim as shown in Table 1. Compared with the control group, HIF-1α and VEGF expression were up-regulated in the $CoCl_2$ group ($P<0.05$). HIF-1α and VEGF expression were down-regulated in the $CoCl_2$+Sim group compared with the $CoCl_2$ group ($P<0.05$).

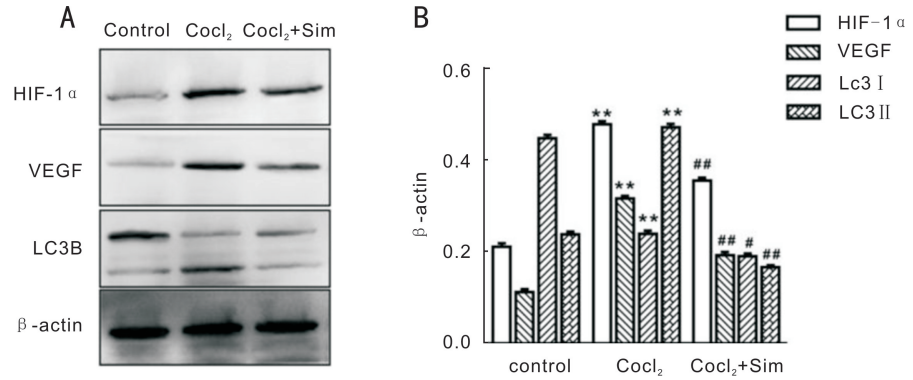

Figure 3 Effects of Sim on the expression of HIF-1α, VEGF, and LC3B in RPE-19 cells A: Western blot was used to detect the expression in each group (control group, $CoCl_2$ group and $CoCl_2$+Sim group); B: The grayscale ratios of HIF-1α, VEGF and LC3B (LC3II/LC3I) to the internal reference β-actin in 3 replicates. $^{**}P<0.01$ *vs* control; $^{\#\#}P<0.01$ *vs* $CoCl_2$ group; $^{\#}P<0.05$ *vs* $CoCl_2$ group. Sim is short of simvastatin.

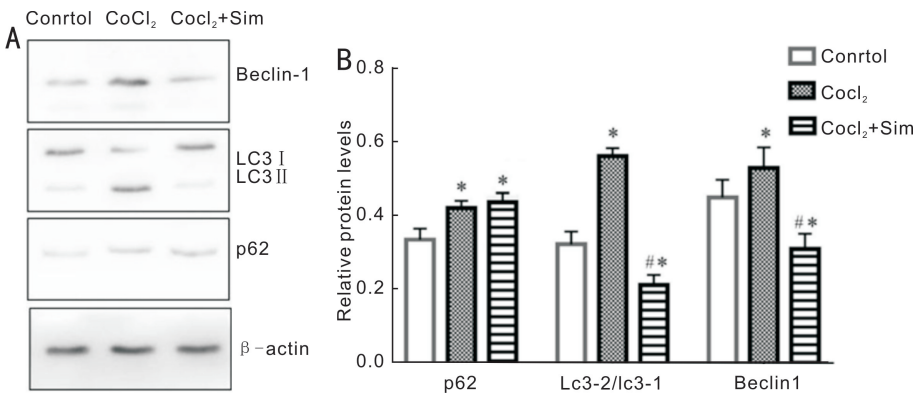

Figure 4 Effects of Sim on the autophagy of RPE-19 cells A: The effect of Sim on autophagy was detected by Western blot assay; B: The grayscale ratios of autophagy-related proteins to the internal reference β-actin in 3 replicates. $^{*}P<0.05$ *vs* control; $^{\#}P<0.05$ *vs* $CoCl_2$ group. Sim is short of simvastatin.

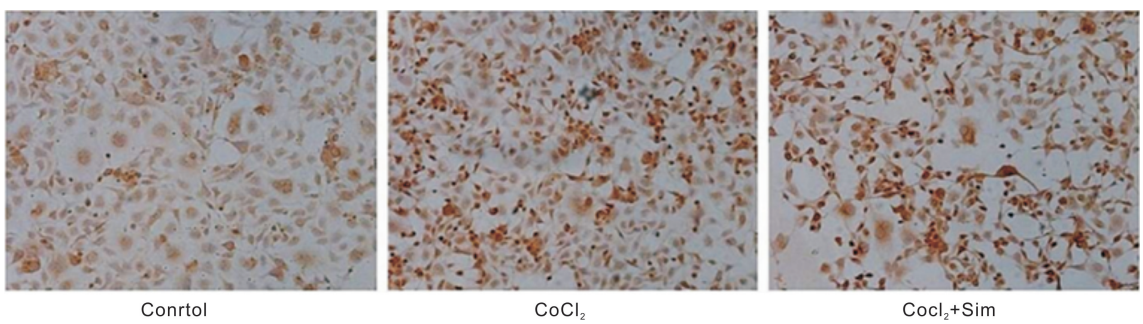

Figure 5 Comparison of apoptotic cells in each group (Tunel dyeing) Sim is short of simvastatin.

Sim Inhibited the Expression of Autophagy Protein in Cells in RPE Cells Under Hypoxia Autophagy level was monitored by expression of autophagy related proteins Beclin-1, LC3B, and p62. RPE-19 cells were lysed and processed for determination of LC3B-II/LC3B-I ratio, p62, and Beclin-1 by Western blot method, and it was found that autophagy related proteins were expressed in all of the three group, but the expression level of P62, Beclin1, and LC3B proteins in the Sim group was lower than the $CoCl_2$ group but higher than control, and the difference was statistically significant (Figure 4).

Effects of Sim on the Cell Apoptosis in RPE Cells Under Hypoxia The TUNEL assay was used to detect the effect of apoptosis on cell apoptosis: genomic DNA would be broken when the cell apoptosis occurred. The TUNEL assay could mark the exposed 3-0h, and the cell apoptosis could be observed with the light microscope. The results were shown in Figure 5. Under hypoxia, Sim promoted the apoptosis of RPE cells.

DISCUSSION

Hypoxia plays an important role in leading the progression of ARMD. In our study, we found that Sim restrained the proliferation of RPE cells and inhibited HIF-1α and VEGF expression under hypoxia. Our results suggested that Sim inhibited autophagy and promoted apoptosis in RPE cells under hypoxia. Activation of autophagy protects cells and reduces apoptosis under stress conditions. Our results suggested that the mechanism by which Sim promoted

apoptosis in RPE cells may be related to its inhibition of autophagy.

It is known that multiple factors affected the progression of ARMD. The function of statins in the ARMD development has been researched in many clinical and epidemiological types of studies[5-8]. Statins are well tolerated and severe side effects are rare[12]. Previous studies have shown that statins are a potentially effective drug in ARMD[13]. Statins are divided into two categories: lipophilic drugs and hydrophilic drugs[14]. This classification is important because different lipophilic statins may have different effects. Lipophilic statins (such as atorvastatin) are more effective than hydrophilic drugs (such as pravastatin) in reducing apoB100 secretion and cholesterol level in cultured RPE cells by regulating RPE cholesterol levels[15]. Statins have therapeutic effect on dry ARMD. A study found that high-dose atorvastatin can induce drusen degeneration, no atrophy or neovascularization, and improved the vision of high-risk subgroup of ARMD patients[16]. However, the precise mechanism of the therapeutic effect of statins is still not clear. It was reported that lipotropic statins such as atorvastatin and Sim also promoted the phagocytic function of RPE cells[17]. Statins have been reported to significantly reduce the risk of early ARMD by about 13%. This is mainly due to the reduction of serum LDL cholesterol levels by statins, which in turn reduces the deposition of LDL cholesterol in drusen[18]. Statins also reduce the plasma concentration of C-reactive protein (CRP) by decreasing the number and function of inflammatory cells[19]. As an inflammation maker, a high concentration of CRP inhibits the inactivation of the complement cascade, leading to dysfunctions in RPE cells and accumulation of drusen[20]. Therefore, statins might play a more important role in the initiation of drusen and delay the onset and progression of early ARMD.

Some studies have shown that statins may be involved in the regression of pigment epithelial detachments and helped to improve vision[17]. Atorvastatin has been proved to have multiple effects on regulating various biological behavior of human RPE cells. It was reported that statins inhibited cell proliferation and migration[21]. It has been reported that atorvastatin and Sim reduced oxidative stress-induced RPE damage, which is a factor affecting the pathogenesis of ARMD and improving the activity of RPE cells[22-23]. Besides, statins may also play a role in neovascularization, and participate in the pathogenesis in wet ARMD either. It is reported that atorvastatin inhibited laser-induced CNV effectively and decreased the inflammatory chemokine and VEGF[24], prompting statins may also prevent the progression from dry ARMD to wet ARMD. Other studies also found that atorvastatin inhibited CNV and down regulated VEGF expression[12]. However, some other studies have shown that Sim promoted VEGF expression and neovascularization in vascular endothelial cells[11]. In our study, we found that Sim inhibited the levels of HIF and VEGF protein, which might be the mechanism of Sim under wet ARMD treatment.

Statins were supposed to be participated in the regulation of HIF-1α. We have previously found that the inhibition of autophagy down-regulated the expression of VEGF[25]. Therefore, we speculate that the inhibition of VEGF expression by Sim may be related to autophagy either. Researches have found that cholesterol loading in hepatocytes lead to the activation of HIF-1α. This is due to hypoxia and the excessive production of nitric oxide and mitochondrial reactive oxygen species[26]. Hisada *et al*[27] have revealed that fluvastatin attenuated HIF-1-dependent ET-1 gene expression in human vascular smooth muscle cells (VSMC) *in vitro*. Our study also found that after treatment with Sim, the expression of HIF-1α was consistent with the reduction of VEGF protein expression in time. It is known that VEGF is a downstream regulator of HIF-1[28-29]. Therefore, we speculated that Sim might downregulate VEGF protein by inhibiting the expression of HIF-1α. This may be one of the important mechanisms of statins preventing CNV progression. Huang *et al*[11] found that statins regulate the expression of HIF-1α through the RhoA pathway after vascular endothelial cell injury, thus increasing the expression of VEGF. Similar studies have also shown that statins can regulate the expression of HIF-1α, but some results displayed down-regulated while others were up-regulated. For example, in the vascular endothelial injury model, statins may up-regulate the expression of HIF-1α, reduce the oxidative stress response, and the expression of VEGF, Akt, and eNOS[30]. Besides, statins can effectively inhibit the expression of HIF-1α, reduce the expression of VEGF and phosphorylated STAT3, and control the expression of ICAM-1 in vascular endothelium[27,31]. All of these studies indicate that statins play a biological role by regulating the expression of HIF-1α. In brief, we indicate that Sim involves in hypoxia signaling in cultured RPE cells. Sim effectively reduces cell proliferation and HIF-1α and VEGF protein expression *in vitro*. It is reasonable to propose that Sim potentially provides a means to attenuate the damage of ARMD. Further studies still need to be conducted to reveal the exact molecular mechanism of Sim treatment for ARMD *in vitro* and *in vivo*.

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