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Effect of acetyl L-carnitine on human retinal pigment epithelium-19 cells in hypoxic conditions

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乙酰左旋肉碱在缺氧条件下对人视网膜色素上皮-19 细胞的影响

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摘要

目的:使用缺氧模型探究乙酰左旋肉碱(ALCAR)对人视网膜色素上皮(ARPE-19)细胞活力、形态完整性和血管内皮生长因子(VEGF)表达的影响。

方法:第一组实验通过暴露 ARPE-19 细胞培养物于不同浓度确定最佳 $CoCl_2$ 剂量。建立五组 ARPE-19 细胞培养物,包括对照组,假手术组(200 μM $CoCl_2$)和分别接受 1、10、100 mM ALCAR 联合 200 μM $CoCl_2$ 组,以评估 ALCAR 对细胞活力的影响。使用 MTT 法测量细胞活力。通过倒置相差显微镜观察细胞的形态特征。采用酶联免疫吸附实验(ELISA)检测 ARPE-19 细胞分泌 VEGF 和 HIF-1α 的水平。

结果:ARPE-19 细胞暴露于不同剂量的 $CoCl_2$ 中,以创建缺氧模型。然而,暴露于浓度为 200 μM $CoCl_2$ 时,细胞活力显著降低至 83%。ALCAR 在 1 mM 和 10 mM 浓度下可增加细胞活力,而最大浓度(100 mM)没有额外效果。与假手术组相比,浓度为 1 mM 和10 mM ALCAR 组的细胞活力显著更高($P=0.041$、0.019)。细胞活力和形态不受最大剂量 ALCAR (100 mM)的影响。与假手术组相比,10 mM ALCAR 组 VEGF 和 HIF-1α 水平显著降低($P=0.013$、0.033)。

结论:ALCAR 是可行的治疗选择,可为视网膜疾病开辟新的治疗途径,与年龄相关性黄斑变性(AMD)特别相关。然而,仍需开展进一步研究明确定义其确切机制,以期充分阐明 ALCAR 对抗视网膜疾病的应用潜力。

关键词:乙酰左旋肉碱(ALCAR);人视网膜色素上皮细胞(ARPE-19);血管内皮生长因子(VEGF);缺氧诱导因子(HIF-1α)

Abstract

• AIM: To investigate the effect of acetyl-L-carnitine (ALCAR) on cell viability, morphological integrity, and vascular endothelial growth factor (VEGF) expression in human retinal pigment epithelium (ARPE-19) cells using a hypoxic model.

• METHODS: In the first set of experiments, the optimal $CoCl_2$ dose was determined by exposing ARPE-19 cell cultures to different concentrations. To evaluate the effect of ALCAR on cell viability, five groups of ARPE-19 cell culture were established that included a control group, a sham group (200 μM $CoCl_2$), and groups that received 1, 10 and 100 mM doses of ALCAR combined with 200 μM $CoCl_2$, respectively. The cell viability was measured by MTT assay. The morphological characteristics of cells were observed by an inverted phase contrast microscope. The levels of VEGF and HIF-1α secretion by ARPE-19 cells were detected by enzyme linked immunosorbent assay (ELISA) assay.

• RESULTS: ARPE-19 cells were exposed to different doses of $CoCl_2$ in order to create a hypoxia model. Nevertheless, when exposed to a concentration of 200 μM $CoCl_2$, a notable decrease in viability to 83% was noted. ALCAR was found to increase the cell viability at 1 mM and 10 mM concentrations, while the highest concentration (100 mM) did not have an added effect. The cell viability was found to be significantly higher in the groups treated with a concentration of 1 mM and

10 mM ALCAR compared to the Sham group (P= 0.041, P= 0.019, respectively). The cell viability and morphology remained unaffected by the greatest dose of ALCAR (100 mM). The administration of 10 mM ALCAR demonstrated a statistically significant reduction in the levels of VEGF and HIF-1α compared with the Sham group (P= 0.013, P= 0.033, respectively).

• CONCLUSION: The findings from the current study indicate that ALCAR could represent a viable therapeutic option with the potential to open up novel treatment pathways for retinal diseases, particular relevance for age-related macular degeneration (AMD). However, to fully elucidate ALCAR's application potential in retinal diseases, additional investigation is necessary to clearly define the exact mechanisms involved.

• KEYWORDS: acetyl-L-carnitine (ALCAR); human retinal pigment epithelium (ARPE-19); vascular endothelial growth factor (VEGF); hypoxia-inducible factor 1 (HIF-1α)

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INTRODUCTION

Age-related macular degeneration (AMD) poses a significant threat to vision, especially in developed countries where human life expectancy is prolonged[1]. Unfortunately, there is not yet a definitive treatment to stop the progression of AMD. Given that inflammation and oxidative stress are involved in the pathophysiology of AMD, efforts have been underway to discover innovative treatments for the condition[2]. The retina has a high metabolic activity, which results in a high oxygen consumption rate per unit. Retinal cells undergo permanent cell loss after being subjected to a cascade of degenerative processes when repeatedly exposed to ischemia and oxidative stress[3-4].

Hypoxia is regulated by hypoxia-inducible factor (HIF), an oxygen-dependent transcription factor consisting of two subunits[5]. HIF-1α, the oxygen-sensitive subunit, is stabilized under hypoxic conditions and degraded under normal oxygen conditions. Additional factors, proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), have been observed to potentially facilitate the expression of HIF[6]. When HIF-1α accumulates in the nucleus, it triggers the release of vascular endothelial growth factor (VEGF) mRNA, leading to new vessel development and having a mitogenic effect on other cells[7]. Studies have demonstrated that hypoxia considerably enhances the expression of HIF-1α and that a decrease in HIF-1α in the vitreous reduces the expression of VEGF[8]. Choroidal neovascularization (CNV) is currently treated using anti-VEGF medicines[9]. However, there is potential for medications that specifically target the HIF pathway to offer improved safety and efficacy[10].

The ARPE-19 cell line is frequently used to study hypoxia-induced retinal neovascularization, with cobalt(II) chloride ($CoCl_2$) employed as a chemical agent to simulate hypoxic conditions in these cells. This approach is crucial for understanding the effects of hypoxia on retinal pigment epithelial (RPE) cells and evaluating potential treatments for conditions such as AMD and retinal neovascularization[11]. Extensive research has been conducted on the correlation between damage to RPE cells caused by hypoxia and the expression of VEGF and HIF. In RPE cells, it has been observed that the levels of mRNA and protein for HIF-1α increase when exposed to hypoxia, which is then followed by an increase in VEGF expression[12-14].

L-carnitine, which aids in energy metabolism and has antioxidant properties, may inhibit inflammatory-induced angiogenesis[15]. Acetyl-L-carnitine (ALCAR), a short-chain ester derivative of L-carnitine, plays a strategic role in mitochondrial functions and has been shown to inhibit angiogenesis[16-17]. The anti-inflammatory and antioxidant activities of ALCAR have been extensively documented in previous research[18-19]. Furthermore, recent investigations have demonstrated its anti-VEGF properties in cancer cells[15,17].

Our study aimed to assess the potential efficacy of ALCAR as a treatment for AMD, a condition that currently lacks a definitive and effective treatment. In this study, we conducted an investigation on the effects of ALCAR on the reduction of VEGF and HIF-1α expression, as well as its impact on cell survival and morphology. The human retinal epithelial cells (ARPE-19) were utilized as the experimental model for this evaluation. To our knowledge, this is the first study to highlight the ability of ALCAR to decrease VEGF and HIF-1α expression *in vitro* in ARPE-19 cells.

MATERIALS AND METHODS

Ethical Approval The ethical aspects of this study were reviewed and approved by the Non-Invasive Research Ethics Board of Fırat University, which is in compliance with international guidelines for human research protection (IRB reference number: 97132852/050.04).

Chemicals and Reagents Buffers and other compounds, such as $CoCl_2$, ALCAR, potassium hydroxide, sodium hydroxide, and 3-[(3-cholamidopropyl) dimethylammonio] The following ingredients were purchased from Sigma-Aldrich (St. Louis, MO, USA): 1, 1, 3, 3-tetraethoxypropane (TEP), tris(hydroxymethyl) aminomethane, Triton X-100, and ethylene glycol-bis(2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA). We bought all our organic solvents from Merck in Darmstadt, Germany. The American Type

Culture Collection (Manassas, VA) provided the ARPE-19 cell line for use in research. Lonza (Atlanta, GA) provided Dulbecco's Modified Eagle Medium F-12 (1∶1 mix) (DMEM/F-12). Fisher Scientific (Pittsburgh, PA) provided the fetal bovine serum, Hyclone provided the penicillin/streptomycin solution, and MP Biomedicals (Santa Ana, CA) provided the 1X trypsin-EDTA. Trevigen provided the Greiner Bio-One MTT Cell Proliferation Assay Kit, and Fisher Scientific (Pittsburgh, PA) provided the dimethyl sulfoxide. Enzyme linked immunosorbent assay (ELISA) kits for human VEGF and HIF-1 were purchased from Abcam in Cambridge, Massachusetts, USA.

Cell Culture and Treatment To keep ARPE-19 cells alive at 37 ℃ with 5% CO_2, streptomycin (100 mg/mL), penicillin (100 U/mL), and DMEM/F12 supplemented with 10% FBS were employed. All treatments were initiated when the cells were 80% or more confluent. The tests were conducted on cells in passages 3 through 10. To encourage cell attachment, 96-well plates were seeded with 10 000 cells per well and then incubated for 24 h at 37 ℃ with 5% CO_2. Five groups of ARPE-19 cell cultures were created to determine the ideal $CoCl_2$ dose in the hypoxic scenario. The first group was the control group, and 100, 150, 200, and 250 μM $CoCl_2$ were administered to the other groups, respectively, over the course of 24 h. The MTT test was used to assess cell viability in ARPE-19 cells. We chose 200 μM $CoCl_2$ as the study starting point because this concentration was found to exhibit the hypoxic pattern.

Study Groups and MTT Assay Group I was the control; cells were cultured for 24 h in its own growth medium. The cells in Group II were exposed to 200 μM $CoCl_2$ for 24 h. $CoCl_2$+ 1 mM ALCAR was used in Group III, and the cells were cultured for 24 h with 200 μM $CoCl_2$ and 1 mM ALCAR. $CoCl_2$+ 10 mM ALCAR group was in Group IV, and the cells were cultured with 200 μM $CoCl_2$ and 10 mM ALCAR for 24 h. Group V was incubated with 200 μM $CoCl_2$ and 100 mM ALCAR for 24 h.

Using the MTT assay, we determined how ALCAR affects cell viability. After aspirating the medium, cells were treated with the MTT reagent for 3 h at 37 ℃ and 5% CO_2. Each well received 150 mL of 100% DMSO to dissolve the formazan salt after the yellow medium was aspirated. By measuring absorbance at 570 nm and making the following calculations, the number of viable cells was determined. Cell viability (%) = [(absorbance of the test sample-absorbance of the control sample)/mean absorbance of the control ×100].

Vascular Endothelial Growth Factor and Hypoxia-Inducible Factor-1α Analysis of VEGF secretion by ARPE-19 cells in a chemically hypoxic environment. ARPE-19 cells were grown in 6-well plates at 105 cells per well. The conditioned medium was removed after 24 h, centrifuged for 5 min at 150 g, and the supernatants were put in vials and kept at -80 ℃ for further investigation. According to the VEGF and HIF-1α protein criteria, measurements in conditioned medium were taken in human VEGF and HIF-1α kits. A microplate reader, Synergy H4 (Biotek Industries, Inc., Winooski, VT) was used to read absorbance values (450 nm). The standard curve was used to calculate VEGF and HIF-1α workouts.

Cellular morphological parameters were observed after therapy. After making the appropriate preparations, we used an inverted phase contrast microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) to take pictures at 100X magnification.

Statistical Analysis Data were expressed as mean±SD, with statistical analyses conducted using SPSS (version 15.01, SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U* test was employed to determine the significance of differences between treatment groups, with a *P*-value of less than 0.05 considered statistically significant.

RESULTS

High $CoCl_2$ Concentrations Reduce Human Retinal Epithelial Cells-19 Cell Viability To determine the best concentration for further research, ARPE-19 cells were subjected to $CoCl_2$ concentrations between 100 and 250 μM for 24 h. When $CoCl_2$ concentration was raised above 200 μM, it was seen that cell viability dropped to 83% (Figure 1). Cells are viable in accordance with the ISO 10993-5 standard when their viability is greater than 80%[20]. Considering these findings, we decided to use 200 μM of $CoCl_2$ in subsequent research.

Acetyl-L-Carnitine Reduces $CoCl_2$-induced Cytotoxicity In human RPE cells exposed to 200 μM $CoCl_2$, we tested the effects of ALCAR at concentrations of 1, 10, and 100 mM on $CoCl_2$-induced cytotoxicity. When measured using the MTT assay and compared to $CoCl_2$-treated cells without ALCAR, the lowest concentrations of ALCAR (1 mM and 10 mM) increased cell viability (P=0.041,0.019, respectively). Cell viability was unaffected even at the highest dose of ALCAR (100 mM; Figure 2).

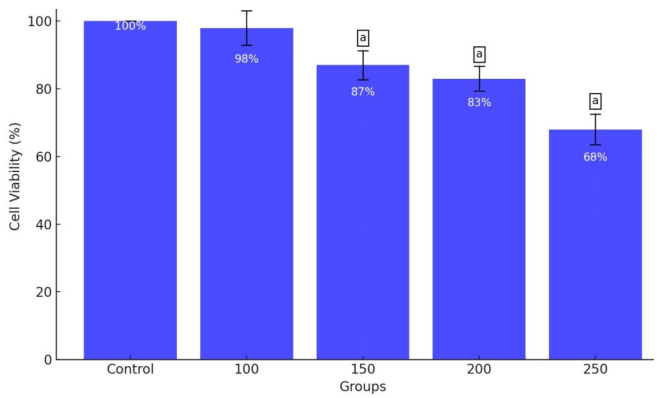

Figure 1 The results of cell viability analysis conducted after 24 hours of exposure to different concentrations of $CoCl_2$ (100, 150, 200, and 250 μM) compared with the control. [a] $P<0.05$ *vs* control, data are presented as the mean±SD (n=6).

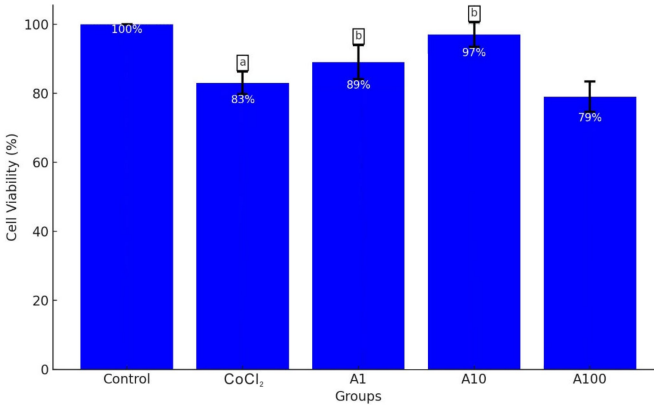

Figure 2 Effects of acetyl－L－carnitine on cell viability in hypoxia model of human retinal epithelial cells. $^{a}P<0.05$ *vs* Control $^{b}P<0.05$ *vs* $CoCl_2$. Different concentrations of acetyl-L-carnitine were tested: 1 μM (A1), 10 μM (A10), and 100 μM (A100). Data are presented as the mean±SD ($n=6$).

Acetyl－L－carnitine Preserved its Cell Morphology and Viability Due to financial constraints, we were unable to perform EdU staining in our study, which consequently limited our ability to compare cell viability numbers directly. Furthermore, considering the well-tolerated nature of ALCAR and its broad dosage safety range, we decided not to utilize the 1 mM ALCAR group in these experiments. This decision was based on the premise that higher concentrations would more effectively demonstrate the potential effects and tolerability of ALCAR, aligning with the compound's known safety profile. Therefore, our study focused on evaluating the effects of ALCAR at higher concentrations to better understand its impact under the specified conditions. When the images of cells in ALCAR－treated groups were compared with the hypoxia model, an increase in cell number and viability was observed with the low dose of ALCAR. However, it was observed that the 100 μM dose of ALCAR had a negative effect on the cell number (Figure 3). High－density cell proliferation was observed in panels A and C. This implies that the cells are growing vigorously and healthily. There is a discernible decrease in the quantity of cells and structural changes in panels B and D. A lower degree of confluence and a more dispersed arrangement were seen in the cells, and some of them took on rounder shapes and unusual sizes. These findings suggest that there may be a negative interaction with the cells or that there may be a hazardous chemical exposure. Apoptosis can be initiated or triggered by cellular stress, as indicated by morphological changes. To avoid toxic effects on ARPE－19 cells, concentrations of less than 100 μM were used in further studies.

The levels of VEGF and HIF-1α secretion in ARPE-19 cells were measured using ELISA. This assessment was conducted under standard settings, using a hypoxic model and comparing the effects of therapy with 1 and 10 mM ALCAR (Table 1). With 200 μM concentrations of $CoCl_2$, induced chemical hypoxia led to significantly higher VEGF and HIF-1α ($P=0.021$ and $P=0.014$, respectively).

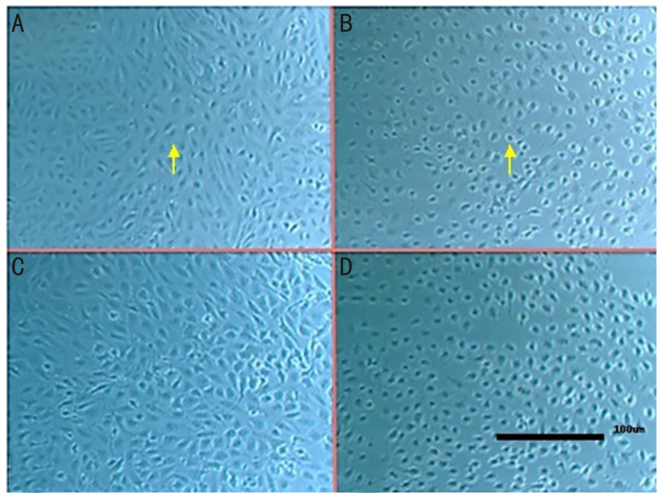

Figure 3 Cell morphology under hypoxic conditions with acetyl－L－carnitine treatment. Panel A (control group) shows high-density cell proliferation, with the yellow arrow pointing to a cell exhibiting typical morphology, indicative of healthy growth. Panel C ($CoCl_2$ and 10 mM acetyl-L-carnitine exposure) also shows similar cell density and health. In contrast, panel B, with 200 μM $CoCl_2$ exposure, and panel D, with $CoCl_2$ and 100 mM acetyl-L-carnitine exposure, reveal notable morphological changes; the yellow arrow in panel B specifically indicates a cell that has undergone morphological alterations.

VEGF was secreted 2.4 times more in hypoxic conditions than in normoxic culture media. According to Table 1, VEGF levels decreased after administration of ALCAR concentrations of 1 mM and 10 mM ($P=0.047$ and $P=0.013$, respectively), confirming ALCAR's ability to control VEGF secretion. HIF－1α secretion increased five times more in hypoxic conditions compared to normoxic culture media. HIF-1 levels decreased following administration of ALCAR doses of 1 mM and 10 mM, as shown in Table 1 ($P=0.17$ and $P=0.033$, respectively). Comparing the ALCAR－1 group to the sham group, the decrease was not statistically significant.

DISCUSSION

The results indicate that ALCAR, which has been shown to suppress HIF－1α in previous cancer studies, also has a similar effect on ARPE－19 cells. This suggests that ALCAR has the potential to be used as a therapeutic drug in retinal diseases where hypoxia plays a role in the development of the condition[15]. On the basis of recent research indicating the capacity of ALCAR to suppress both VEGF and HIF－1 in a variety of cancer cell lines, we think that it could be a possible treatment option for wet AMD. The etiology of wet AMD is significantly influenced by complement system mutations connected to apoptosis, persistent inflammation, lipofuscin buildup, and oxidative damage[20-21]. It is said that using methods other than VEGF to impact the pathophysiological process will have a synergistic effect in the therapy of AMD[22].

Most medications used to treat AMD currently have an impact on the VEGF regulation. However, since AMD is a multifactorial

Table 1 Effect of acetyl-L-carnitine on vascular endothelial growth factor and hypoxia-inducible factor-1α in human retinal epithelial 19 cells $\bar{x}\pm s$

Groups	VEGF (pg/mL)	HIF-1α (pg/mL)
Control	285±35.0	240±25.6
Sham	702±71[a]	1270±92[a]
1 mM ALCAR+200 μM $CoCl_2$	370±48[b]	1140±84
10 mM ALCAR+200 μM $CoCl_2$	320±44[b]	620±59[b]

VEGF: Vascular endothelial growth factor; HIF: Hypoxia-inducible factor. [a]$P<0.05$ *vs* Control; [b]$P<0.05$ *vs* Sham.

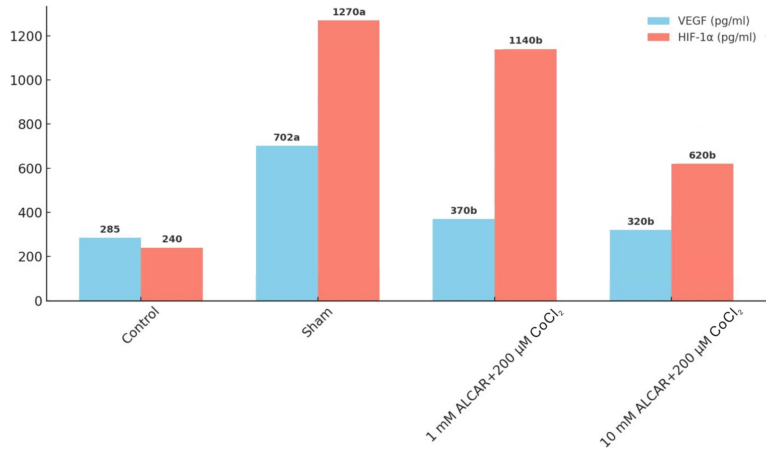

Figure 4 Effect of acetyl-L-carnitine on vascular endothelial growth factor and hypoxia-inducible factor-1α in human retinal epithelial-19 cells. [a]$P<0.05$ *vs* Control; [b]$P<0.05$ *vs* Sham.

disease, treating it solely with an anti-VEGF effect may not be sufficient. The development of geographical atrophy (GA) following long-term anti-VEFG treatment and the potential role of anti-VEFG drugs in this process have been the subjects of several study[23-25]. It was found that long-term intravitreal anti-VEGF medications (Bevacizumab and Ranibizumab) given to patients with wet AMD increased GA in the cases in the analyses of the CATT, IVAN and HARBOR investigations[26-29].

A wide range of ocular pathologies, including AMD, cataracts, glaucoma, dry eye syndrome, eye disorders brought on by systemic disease (diabetic retinopathy, neurotrophic or bullous keratopathy), and hereditary ocular conditions (retinitis pigmentosa, keratoconus), are covered in detail regarding ALCAR food supplements[30]. However, a review of the literature we conducted using Google Scholar and PubMed revealed no studies demonstrating an *in vitro* effect on AMD. Recent research has revealed that ALCAR exerts an antiangiogenic effect through the reduction of VEGF and HIF-1 levels. Given its demonstrated anti-angiogenic and anti-inflammatory properties, ALCAR presents potential for use in the angioprevention of cancer[15]. ALCAR has been demonstrated to cure electroretinographic (ERG) abnormalities in rats with streptozotocin-induced diabetes by considerably increasing b-wave amplitude and its antioxidative actions in retinal ganglion cells with high glucose-induced oxidative stress[31-32].

A common indicator of hypoxia is HIF-1α. As the activation of the enzymes responsible for HIF-1α degradation declines during hypoxia, HIF-1α rapidly accumulates[33]. $CoCl_2$ results in an increase in HIF-1α, resulting in a hypoxic model[34]. The presence of hypoxic circumstances induces an increase in the expression of HIF-1α, which subsequently stimulates the production of VEGF messenger RNA. According to Rigiracciolo *et al*[35], in a hypoxic environment, HIF-1α controls the VEGF gene transcriptional activity. Recent studies have shown a marked increase in HIF-1α expression during retinal angiogenesis and hypoxic conditions. The use of the HIF-1 inhibitor Acriflavine leads to a decrease in HIF-1α and VEGF expressions in the vitreous, which in turn inhibits neovascularization associated with proliferative diabetic retinopathy and other ischemic retinopathies[10,36]. In our investigation, it was found that the ALCAR group's HIF-1α level was statistically substantially lower than that of the control group. The relationship between HIF-1α and VEGF is consistent with other research in the literature[37].

In the hypoxia model made using $CoCl_2$ in ARPE-19 cells, we wanted to demonstrate the anti-VEGF and antioxidant capabilities of ALCAR in the retinal pigment epithelium. To start, we conducted a dosage study of $CoCl_2$ to construct a 200 μM $CoCl_2$ hypoxia model. We discovered that 10 mM was the effective dose in the ALCAR dose study. In the ARPE-19 cells hypoxia instance, the group that received ALCAR showed statistically significant increases in cell viability ($P<0.05$). ALCAR demonstrated a protective effect against hypoxia in the morphological assessment of ARPE-19 cells performed with light microscopy (Figure 2). Due to its high dose confidence interval and ease of toleration, ALCAR can be a safe and effective medicinal molecule[38]. Its antiangiogenic function is strong when used at 10 mM or

20 mg/kg, which has previously been proven to be an effective dose in cell culture tests[15]. The literature indicates that ALCAR does not cause cytotoxicity and promotes viability at doses of 1 and 10 mM, and the cell morphology and MTT tests in this study support this claim.

ALCAR is a chemical having a long history of use as a food supplement, a high dosage confidence interval, and low cost compared to anti-VEGF medications. In our study, we assessed how ALCAR affects viability levels, cell shape, VEGF, and HIF-1α in ARPE cells. In cell culture, 10 mM ALCAR equivalent to 20 mg/kg, was found to have antiangiogenic properties. In patients with wet-type AMD, where pathological angiogenesis is a factor, our findings suggest that ALCAR may be advantageous with a similar impact. In addition to the AREDS combination, novel preparations are used to treat wet and dry AMD by using ingredients such as resveratrol[39].

Our research relies on cell culture. The ELISA approach was used to assess the levels of HIF-1α and VEGF, which have anti-angiogenic capabilities. Due to a funding issue, several techniques (Western blot analysis, EdU staining, *etc.*) and variables (IL, CxCR4, ROS, *etc.*) could not be examined to completely understand the antiangiogenic impact mechanism. Both human studies and animal trials should be used to support this research. If similar results are observed in these studies, it would be advantageous to include ALCAR in the regimens used to treat AMD.

According to the findings that we have obtained, ALCAR may have the potential to play a therapeutic role in the treatment of hypoxic retinal disorders, particularly AMD. In order to verify these findings and investigate the underlying processes by which ALCAR exerts its influence on HIF-1α and VEGF, additional research is required. Regardless, the results of this study point the way toward a fruitful line of inquiry for further research in the field of retinal illnesses and emphasize the possible advantages of utilizing organic compounds such as ALCAR in the treatment of AMD and the potential prevention of vision loss caused by AMD.

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