·Basic Research·

Topical dihydroartemisinin inhibits suture-induced neovascularization in rat corneas through ERK1/2 and p38 pathways

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Abstract

• AIM: To determine if topical instillation of dihydroartemisinin (DHA) inhibits corneal neovascularization (NV) in rats and to investigate the role of the extracellular regulated kinases (ERK) 1/2 and p38 pathways in this process.

• METHODS: Suture-induced corneal NV was produced in rats and the eyes were topically treated with different concentrations of DHA (20mg/L, 10mg/L or 5mg/L) or normal saline 4 times a day for 7 days. The corneal NV was quantified as the proportion of NV area to the whole cornea. Western blot was used to determine the expressions of vascular endothelial growth factor (VEGF) and the phosphorylation status of VEGF receptor-2, ERK1/2 and p38 in the corneas. Immunofluorescent staining was used to determine the expressions of phospho-ERK1/2 and phospho-p38 in the corneal tissues from the eyes treated with 20 mg/L DHA (DHA group) or normal saline (control group).

• RESULTS: The proportion of corneal NV area in the eyes treated with normal saline or DHA at dosages of 20mg/L, 10mg/L or 5mg/L was (23.74± 3.00)%, (15.73± 2.88)%, (19.53± 2.42)%, and (23.38± 2.79)%, respectively. In the eyes treated with 20mg/L or 10mg/L DHA, the corneal NV area was significantly reduced when compared to that in eyes with normal saline (P<0.05). Western blot analyses revealed that 20mg/L DHA significantly inhibited the expressions of VEGF and phospho-VEGFR-2. Both 20mg/L and 10mg/L DHA inhibited the expressions of phospho-ERK1/2 and phospho-p38. Immunofluorescent staining further demonstrated that 20mg/L DHA lowered the expression levels of phospho-ERK1/2 and phospho-p38 in the corneas with suture-induced NV.

• CONCLUSION: Suture-induced NV in rat corneas was significantly inhibited by topical treatment with 20mg/L and 10mg/L DHA. The results suggest that the effects could be partially dependent on the DHA-mediated inhibitions of the ERK1/2 and p38 pathways.

• KEYWORDS: cornea; neovascularization; dihydroartemisinin; extracellular regulated kinases 1/2; p38

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INTRODUCTION

Corneal neovascularization (NV) is a sight-threatening condition that is usually associated with inflammatory or infectious ocular diseases [1]. In some instances, corneal NV can be beneficial for clearing infections, wound healing and arresting autoimmune corneal melting [2]. However, corneal NV more frequently leads to scar formation, lipid deposition, immune rejection of corneal grafts and significant visual impairment[3].

The mechanisms of corneal NV have been investigated and vascular endothelial growth factor (VEGF) plays a central role in this process [4,5]. VEGF signaling is mediated mainly through three receptors, VEGFR-1, -2 and -3 [6]. Of these receptors, VEGFR-2 appears to play a more critical role in the regulation of angiogenesis[7,8].

Recently, the role of the mitogen-activated protein kinases (MAPKs) pathways in NV has been a topic of intense study.

MAPKs are a family of well-conserved serine/threonine kinases that control a vast array of physiological processes and mediate many important cellular functions [9]. Extracellular regulated kinases (ERK) 1/2 and p38 are two major members of the MAPKs family that are involved in angiogenesis [10,11]. The activation of VEGFR-2 leads to the activation of ERK1/2, which regulates endothelial cell (ECs) proliferation[12], and of p38, which regulates migration of ECs[13].

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua* [14], is an effective novel anti-malarial drug with low toxicity [15]. Recently, DHA was also shown to inhibit angiogenesis in studies using human umbilical vein endothelial cells [16,17], rat whole embryo cultures [18] and chicken chorioallantoic membranes[17].

In the current study, we examined whether topical administration of different doses of DHA could inhibit suture-induced corneal NV, and determined the role of ERK1/2 and p38 signaling pathways in this process *in vivo*.

MATERIALS AND METHODS

Animals Twenty-four Sprague-Dawley rats, weighing 150-180g, were used for this study. This study was approved by the Experimental Animal Center of Southern Medical University, Guangzhou, China. All animal procedures were conducted in accordance with the statement of Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Visual Research. All rat corneas were carefully checked before the procedure using a slit-lamp microscope and the corneas with any defects or new vessels would be excluded.

Induction of Corneal NV Prior to the surgical procedure, animals were anesthetized with an intraperitoneal injection of 4mL/kg of 100g/L chloral hydrate. After topical application of 5g/L tetracaine, three 10-0 black silk sutures (Alcon, Fort Worth, USA) were surgically placed at midstromal depth, 1.0mm from the limbus. After the procedure, 0.3% ofloxacin ophthalmic solution was applied 3 times a day for 3 days to minimize the risk of infection. The sutures remained in place during the study.

Anti –neovascularization Treatment On the first post-operative day, 24 rats were randomly divided into 4 groups, 6 rats in each group. DHA (Beijing Holley-Cotec Pharmaceuticals Co. Ltd., Beijing, China) was dissolved in normal saline at the concentrations of 20mg/L, 10mg/L and 5mg/L, and adjusted to pH 7.2. Three groups were treated topically with the DHA solution, at the specified concentration, 4 times each day. As a control, one group was treated with eye drops of normal saline. Corneal changes were monitored daily for 7 days using a slit lamp microscope. The Robert formula [19] was used to determine the corneal NV area on the 7th day. The area of the corneal NV region by the whole area of each cornea was calculated. The corneal NV was quantified as the proportion of NV area to the whole cornea [20]. All of the corneas were microscopically examined by the same operator, who was blinded to the identity of the samples.

Western Blot Rats were euthanized using CO_2 asphyxiation for the collection of the corneal tissue. Three corneas from each of the 4 groups were collected for Western blot analysis. The cornea was dissected and homogenized in a cold buffer containing 50mmol/L Tris-HCL (pH 7.5), 150mmol/L NaCl, 1mmol/L EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, a protease inhibitor cocktail (Sigma, Saint Louis, USA) and a phosphatase inhibitor cocktail (KeyGEN, Nanjing, China). The lysate was centrifuged for 15 minutes at 14 000 rpm and protein concentrations in the supernatant were measured using a BCA protein assay kit (KeyGEN, Nanjing, China). An equal amount of total protein from each sample was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour at room temperature with gentle shaking in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) supplemented with 5% nonfat milk. The membranes were then incubated overnight at 4 C with anti-VEGF, anti-phospho-VEGFR-2, anti-phospho-ERK1/2 or anti-phospho-p38 (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA) antibodies. These blots were then incubated with the corresponding HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 hour at room temperature. Signals were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Bedford, MA) and exposed to x-ray film for 2 minutes. Protein bands were quantified by densitometry, and protein loading was normalized with GAPDH.

Immunofluorescence Microscopy Corneas from the rats treated with 20mg/L DHA and those treated with normal saline (3 rats per group) were fixed in 100g/L paraformaldehyde, embedded in paraffin wax and cut into 5-μm sections according to standard procedures. Cornea sections were deparaffinized using xylene and ethanol, washed in PBS for 5 minutes, incubated with 0.25% trypsin (KeyGEN, Nanjing, China) for 20 minutes, washed with PBS three times for 5 minutes each, then incubated with 10% normal goat serum at room temperature for 30 minutes. Sections were incubated overnight at 4℃ with either anti-CD31/anti-phospho-ERK1/2 or anti-CD31/anti-phospho-p38 antibodies. The slides were washed with PBS

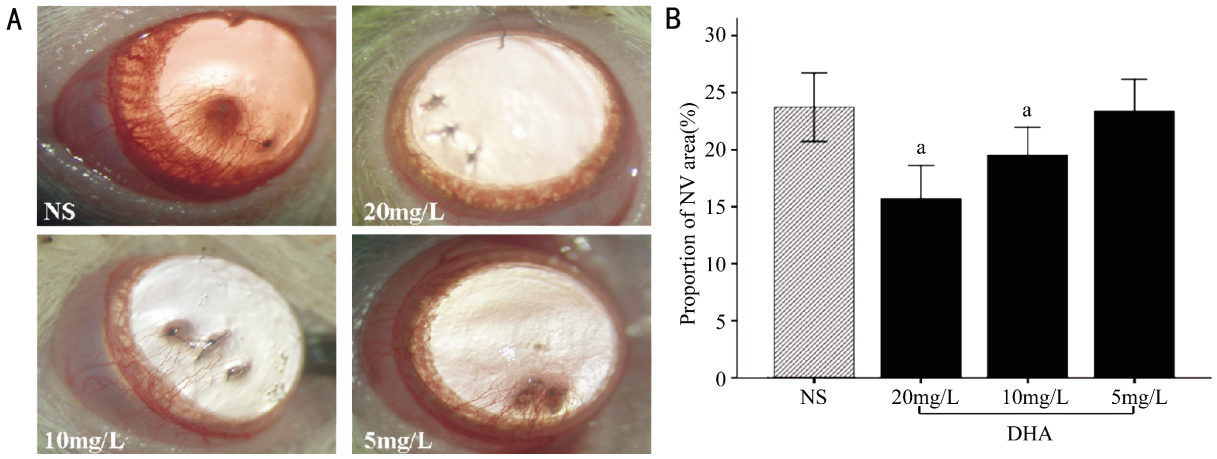

Figure 1 The corneal neovascularization (NV) areas on the 7th day after suture placement A:Photographs showing corneal NV; B: The proportion of NV area to the whole cornea. [a]P<0.05, compared with normal saline group (NS)

three times for 5 minutes each and incubated with Cy3-conjugated anti-goat IgG (Beyotime, Shanghai, China) and Alexa Fluor 488-conjugated anti-rabbit IgG (Zhongshan Goldenbridge, Beijing, China) secondary antibodies at room temperature for 60 minutes. Slides were washed in PBS, incubated with DAPI (Roche, Berlin, Germany) for 5 minutes and mounted. Sections were examined and imaged using a Leica DMI600B microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis All of the statistical analyses were performed using SPSS 13.0. Data are presented as mean ± SD. One-way analysis of variance (ANOVA) test was used to compare the effects of the different DHA concentrations on corneal NV to the controls. Post-hoc analyses were conducted using the least significant difference (LSD) test. A value of P<0.05 was considered as statistically significant.

RESULTS

Inhibition of Suture-Induced Corneal NV with DHA The corneal NV in the normal saline group covered the sutures and even reached the corneal center, but only a minimal number of vessels were found in corneas from the 20mg/L DHA-treated group 7 days after the procedure (Figure 1A). The proportion of NV area to the whole cornea in the normal saline group and the 20mg/L, 10mg/L and 5mg/L DHA-treated groups was 23.74%±3.00%, 15.73%±2.88%, 19.53%±2.42% and 23.38%±2.79%, respectively (Figure 1B). Compared to the saline-treated rats, DHA at 20mg/L and 10mg/L significantly inhibited the cornea NV (P=0.000 and P=0.016, respectively), while 5mg/L DHA group showed no similar effect (P=0.826). Furthermore, treatment with 20mg/L DHA promoted a stronger inhibition than treatment with 10mg/L of DHA (P=0.028).

Expressions of VEGF and VEGFR-2 Phosphorylation For assessment of the effects of DHA on expressions of VEGF and VEGFR-2 phosphorylation in the corneal tissue,

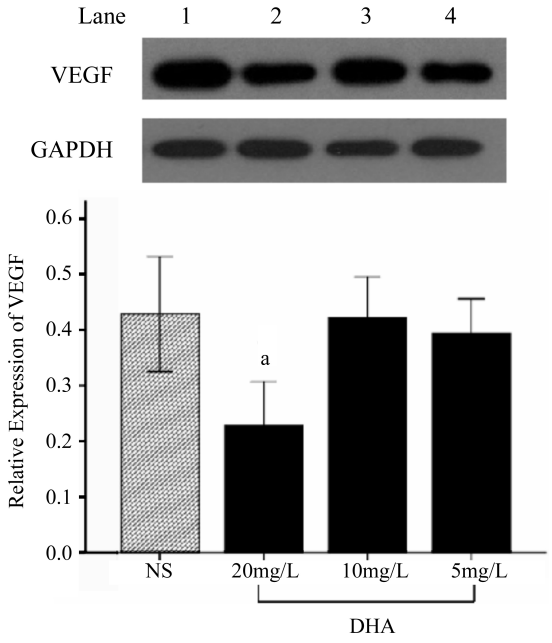

Figure 2 Western blot analyses of VEGF expression [a]P<0.05, compared with normal saline group (NS)

Western blot analyses revealed that 20mg/L of DHA significantly reduced both expressions of VEGF (Figure 2, Lane 2) and the phosphorylation of VEGFR-2 (Figure 3, Lane 2). However, treatment with 10mg/L or 5mg/L DHA failed to induce any appreciable effects on expressions of VEGF and VEGFR-2 phosphorylation (Figure 2, 3, Lane 3, 4).

Expressions of Phosphorylation of ERK1/2 and p38 ERK1/2 and p38 are two important factors involved in angiogenesis and mediate downstream VEGF signals. Therefore, we also investigated the effect of DHA on ERK1/2 and p38 activation in the cornea. Using Western blot analyses and immunofluorescence microscopy, we examined the expressions and localization of phospho-ERK1/2 and phospho-p38 in corneas with or without DHA treatment. In the Western blot analyses, no significant effect

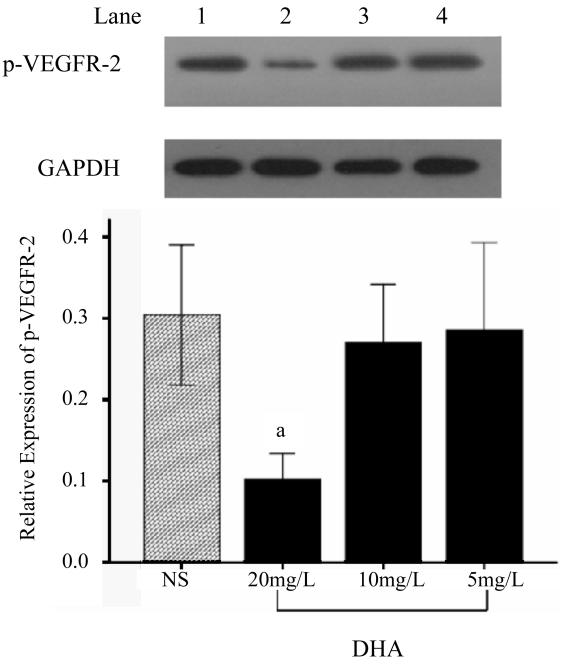

Figure 3 Western blot analyses of phospho -VEGFR -2 expression [a]P<0.05, compared with normal saline group (NS)

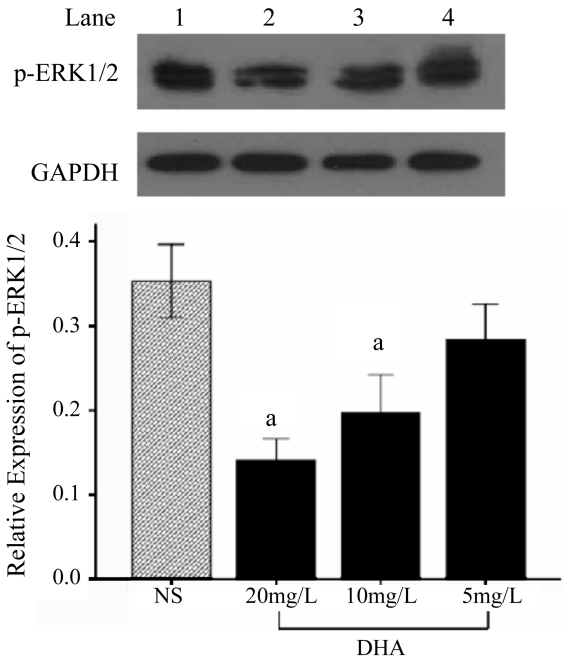

Figure 4 Western blot analyses of phospho -ERK1/2 expression [a]P<0.05, compared with normal saline group (NS)

was observed in the corneas treated with 5 mg/L DHA, while the expressions of phospho-ERK1/2 and phospho-p38 was significantly decreased in the corneas treated with 20mg/L or 10mg/L DHA (Figure 4, 5).

The expressions of phospho-ERK 1/2 (Figure 6) and phospho-p38 (Figure 7) in rat corneas from the 20mg/L DHA-treated group and control group were evaluated using an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (green); the pathological NV was visualized by staining the ECs with Cy3-conjugated anti-goat (red), and the nucleus is indicated by DAPI staining (blue).

Phospho-ERK1/2 (Figure 6A) and phospho-p38 (Figure 7A) were strongly expressed in the corneal epithelium and stromal layers around the corneal suture of control group. While in the eyes treated with 20mg/L DHA, the intensity of expressions for both phospho-ERK1/2 and phospho-p38 was reduced compared to control group (Figure 6B, 7B). Therefore, immunofluorescence microscopy demonstrated that topical administration of 20mg/L DHA reduced phospho-ERK1/2 and phospho-p38 expressions in corneas with suture-induced NV.

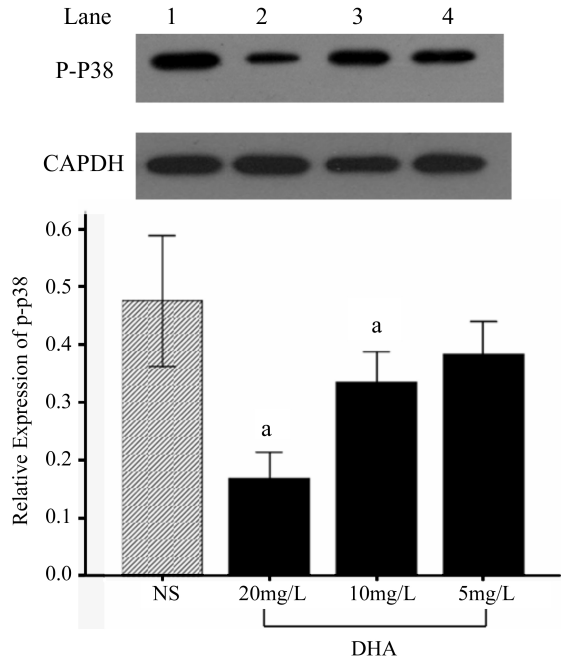

Figure 5 Western blot analyses of phospho -p38 expression [a]P <0.05, compared with normal saline group (NS)

DISCUSSION

Corneal NV is a major public health problem. Although various compounds and methods have been proposed as NV inhibitors in experimental and clinical corneal NV, steroids are still the primary therapy for corneal NV and corneal graft rejection in a clinical setting [2,3]. However, treatment with steroids can often be associated with serious side effects, including cataract, glaucoma and increased risk of infections. As there are only a few options for treating corneal NV, identifying additional therapeutic options is urgently needed.

DHA, which is isolated from the traditional Chinese herb *Artemisia annua*, displays low toxicity[15] and is inexpensive. It is not only an anti-malaria compound but could also inhibit angiogenesis *in vitro* [16,17] and *in vivo* [18]. DHA markedly reduces VEGF binding to its receptors on the

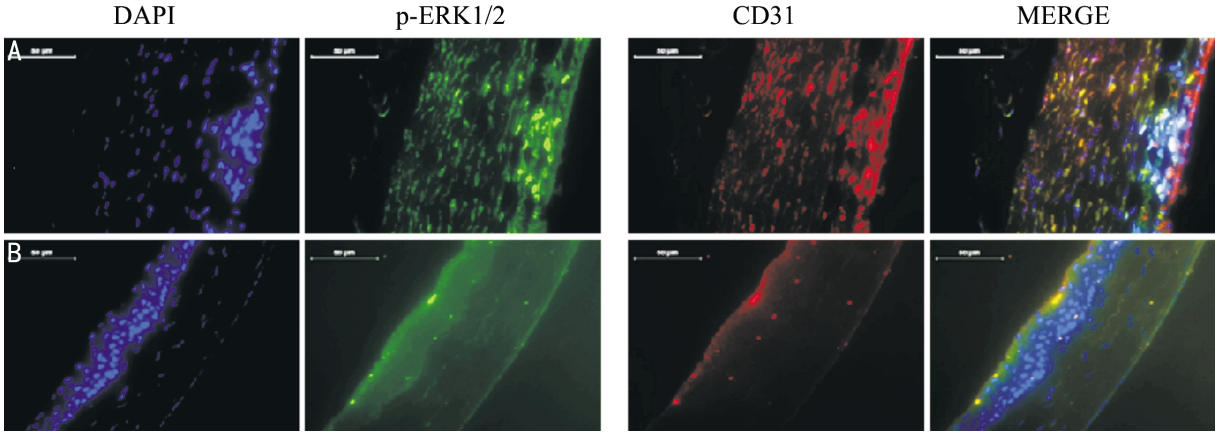

Figure 6 Representative immunofluorescent micrographs showing phospho-ERK1/2 (green), CD31 (red) and the nucleus (blue) in rat corneas from the control group (A) and the 20mg/L DHA-treated group (B) after suture placement The expression of phospho-ERK1/2 and CD31 was found mainly in the corneal epithelium and stromal layers in normal saline group, while only slight phospho-ERK1/2 and CD31 expression was observed in corneal epithelium of the DHA-treated corneas. Scale bar-50μm

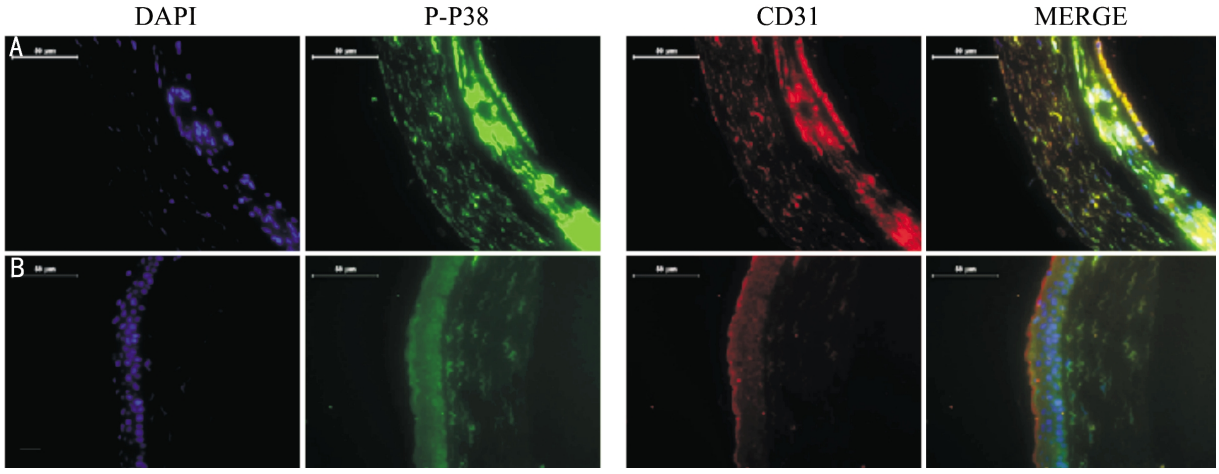

Figure 7 Representative immunofluorescent micrographs showing phospho-p38 (green), CD31 (red) and the nucleus (blue) in the rat corneas from the control group (A) and the 20mg/L DHA-treated group (B) after suture placement Phospho-p38 and CD31 were strongly expressed in the corneal epithelium and stromal layers in the normal saline group. In contrast, phospho-p38 and CD31 were weakly expressed in the 20mg/L DHA-treated group. Scale bar-50μm

surface of human umbilical vein endothelial cells[17].

DHA was a more water-soluble metabolite of artemisinin derivatives [17]. Topical administration of DHA could be a feasible option of the treatment of corneal CV. Therefore, we evaluated if topical administration of different concentrations of DHA might be anti-angiogenic in a rat model of corneal NV. Our data demonstrated that 20mg/L and 10mg/L DHA could significantly reduce the proportion of NV area to the whole cornea. This inhibition was concentration dependent as 5mg/L DHA failed to reduce NV.

VEGF promotes angiogenesis and is one of the most important growth factors [21]. It has been shown that anti-VEGF antibody treatment represses vessel growth [22]. VEGF and its three receptors all play significant roles in vessel formation. Among these receptors, VEGFR-2 is important for endothelial cell proliferation and migration[10,23]. It is mainly expressed on the surface of vascular and lymphatic endothelial cells [24]. The combination of VEGF and VEGFR-2 mediates complicated functions of great importance. In our study, Western blot analyses revealed that 20mg/L DHA promoted a sharp decrease in VEGF expression, suggesting that DHA might reduce corneal NV by inhibiting this pathway.

Furthermore, we also assessed the corneal expression of phospho-VEGFR-2. Phospho-VEGFR-2 is the active form of VEGFR-2 and mediates the downstream signaling pathway. We found that 20mg/L DHA promoted a decrease in the phosphorylation of VEGFR-2. VEGF regulates proliferation, migration of vessel endothelial cells and enhances vascular permeability mainly via the activation of VEGFR-2, which has been strongly implicated in the regulation of angiogenesis [7,8]. Therefore, we hypothesized that reduced phosphorylation of VEGFR-2 played a

significant role in the DHA-mediated inhibition of corneal NV. However, we found that DHA inhibited the expression of VEGF and phosphor-VEGFR-2 at a concentration of 20mg/L only, though corneal NV was inhibited by treatment with 20mg/L and 10mg/L DHA. We speculate that the inhibitory ability of DHA is dose-dependent and there may be other factors that lead to the inhibition of corneal NV.

Previous reports have shown that activation of VEGF receptors results in the activation of ERK1/2, which is involved in cell proliferation[12]. VEGF also activates the p38 pathway, which is required for mitogenic activity in ECs and has been implicated in cell migration [13]. ERK1/2 and p38 are the two major downstream proteins in VEGF signaling. Western blot analyses and immunofluorescence microscopy demonstrated that DHA treatment reduced their phosphorylation. Furthermore, p38 is a key regulator of inflammatory cytokine expression [9], and our model of corneal NV was based on corneal inflammation secondary to corneal sutures; thus, DHA might also inhibit corneal NV by decreasing the expression of inflammatory cytokines induced through the p38 pathway.

In conclusion, topical application of 20mg/L and 10mg/L of DHA inhibited corneal NV and the data suggest that this is, at least partially, due to the inhibition of the ERK1/2 and p38 pathways. However, there are still some problems left, such as whether topical administration of DHA could do harm to rat corneas and whether there might be other signaling pathways involved in this progress.

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