Effect of ERK inhibitor on corneal neovascularization induced by alkali burn in mice and its mechanism

Manhua Xu1*, Kaiming Li,1* Yanxi Wang,1 Jie Wang,2 Mengtian Bai3 and Gangjing Kang1

Abstract
The objective of this study is to explore the effect of extracellular signal–regulated kinase (ERK) inhibitors on corneal neovascularization induced by alkali burn in mice and its mechanism. A total of 30 standard diet (SD) healthy mice were divided into normal group, alkali burn group, and inhibitor group. Normal group was not treated. Alkali burn group and inhibitor group were used to establish corneal neovascularization model induced by alkali burn. After successful modeling, ERK inhibitor was used to intervene in inhibitor group, and saline of equal volume was used in normal group and alkali burn group. The area of corneal neovascularization was calculated and the expression of vascular endothelial growth factor (VEGF), c-Fos, c-Jun, ERK1/2, and p-ERK1/2 protein in cornea tissue of three groups of mice was detected. The relative expression of vascular area, length, VEGF, c-Fos, c-Jun, ERK1/2, and p-ERK1/2 protein in cornea tissue of mice in alkali burn group was significantly higher than that in normal group and inhibitor group. The relative expression of vascular area, length, VEGF, c-Fos, c-Jun, ERK1/2, and p-ERK1/2 protein in cornea tissue of mice in inhibitor group was higher than that in normal group, and the expression level of PEDF was lower than that in normal group (P < 0.05). ERK inhibitors inhibit the formation of corneal neovascularization by inhibiting the expression of VEGF, c-Fos, and c-Jun proteins through the action of ERK signaling pathway.

Keywords
alkali burns, corneal neovascularization, ERK inhibitors

Date received: 7 February 2019; accepted: 21 May 2019

Introduction
The premise of the normal physiological function of the corneal is to ensure its transparent feature, but under some pathological conditions such as infection and chemical damage, it will lead to the formation of corneal neovascularization (CNV), in which the alkali burns are the most serious, and the alkali burns will destroy the corneal tissue, causing the occurrence of the CNV, affecting the normal function of the cornea, and causing losing transparency of the cornea. Thereby, it will affect the visual function and seriously cause loss of visual function.1,2 At present, the treatment of the CNV is mainly to promote degeneration of the neovascularization and anti-angiogenesis, and hormone drugs are often used for treatment, but the effect is not good, which
can easily cause adverse reactions. Extracellular signal–regulated kinase (ERK) inhibitor is an extracellular regulatory protein kinase inhibitor that inhibits the vascular formation promotion factor released by endothelial cells and acts on the ERK/p-ERK signal pathway and thereby inhibits the formation of new neovascularization. In this research experiment, a neovascularization model of alkali-induced burn mice is established to investigate the effects and mechanism of ERK inhibitors on the CNV induced by alkali burns in mice.

Materials and methods

Materials

**Research animals.** A total of 30 standard diet (SD) healthy mice, provided by Shanghai Chao Rui Biological Technology Co., Ltd., were used in this study. They are aged 6–8 weeks, with the average age of 7.2 ± 0.1 weeks. They weighed 20–30 g, with the average weight of 24.0 ± 0.5 g. The raising temperature was 22°C–25°C, and the indoor humidity was 35%–40%. The raising room is regularly sterilized with ultraviolet radiation. They are supplied the uniform standard feed and allowed to move freely, and the feeding time is 1 week. There is no statistical difference in age and weight (P > 0.05) in all mice and it is comparable. All the experiments in this research have been approved by the association of ethics.

**Main drugs and reagents.** ERK inhibitor PD98059 (purchased from CSNpharm), rabbit anti-human vascular endothelial growth factor (VEGF) antibody (purchased from Xiamen Research Biotechnology Co., Ltd.), mouse anti-rat c-Fos and c-Jun antibodies (all purchased from Shanghai Kang Lang Technology Co., Ltd.), rabbit anti-mouse ERK1/2 and p-ERK1/2 antibodies (all purchased from Shanghai Yu Bo Biological Technology Co., Ltd.), enzyme-linked immunosorbent assay (ELISA) test kit (Shanghai National Pharmaceutical Group Chemical Test Co., Ltd.), hematoxylin and eosin (HE) stain, and phosphate-buffered saline (PBS) buffer (Wuhan PhD Bioengineering Co., Ltd.) are the main drugs and reagents used in this study.

Methods

**The group and model establishment**

The 30 healthy SD mice are randomly divided into normal group, the alkaline burn group, and the inhibitor group, with 10 mice in each group; 20 mice in the alkali burn group and the inhibitor group are established as the model of neovascularization induced by alkali burns, and pentobarbital was injected into the abdominal cavity for anesthesia before modeling the mice. Then, for the surface anesthesia of the right eye of the mice, 0.5% proparacaine hydrochloride is dropped into the right eye of the mice. After preparing 1 μL NaOH solution with 1 mol/L, it was dripped on a single-layer circular filter paper of 2 mm diameter. With the help of the surgical microscope, the single-layer circular filter paper dripped of NaOH solution was gently stuck to the right eye of the mice. Selecting the center of the mice right corneal, the cautery treatment is performed for approximately 30 s. After completing the cautery, 0.9% saline solution was used to fully wash the mice’s eye surface and eyelids for about 1 min, and the modeling of CNV induced by alkaline burns was performed. No treatment is performed on the left eye of two groups of mice during the modeling. No treatment is performed on the normal group during modeling.

**Drug interventions**

Three groups of mice were supplied with drug for interventions after successful modeling. The mice in the inhibitor group were treated with ERK inhibitors, and ERK inhibitor PD 98059 was used for mice’s intraperitoneal injection at a dose of 10 mg/kg. Mice in both the normal group and alkaline burn group were injected with the same volume of saline. The mice were allowed to have food freely during the treatment with the drug. All three groups of mice were continuously treated for 14 days, and the effects of intervention were observed.

**Detect the area and length of CNV in mice after modeling for 7 days**

The photographic treatment on the anterior segment in three groups of mice was performed. IPP6.0 was adopted to analyze the length of mice’s CNV (the selected neovascularization was the longest growth and minimum curvature from the corneal margin to the center). Area formula is \( A = C/12 \times 3.1416(r^2 - (r - l)^2) \), in which, A represents the area of mice’s CNV, C represents the number of corneal circular clocks involved in mice neovascularization, R represents the corneal...
radius of mice, and 1 represents the length of mice’s neovascularization.

**Slice and staining**

After 14 days of continuous treatment in the three groups of mice, the eyeballs of mice in normal group and eyes induced in alkali burn group and the inhibitor group were removed, and the paraffin slices were prepared and continuously sliced into the size of 4 μm. The three groups of mouse slices were stained. The xylene was used for regular dewaxing, 5 min each time, and then the slices were treated with water flash under gradient alcohol for 3 min and rinsed in running tap water for one time. Hematoxylin was used to stain the slices for 5 min, and again the slices were rinsed in running tap water for one time. Ethanol gradient was used for dehydration treatment. Finally, neutral gum was used to seal the film. An optical microscope was used to analyze the image and observe the morphology and number of the neonatal vascular in mice.

**Detecting VEGF level of corneal tissues in three groups of mice**

Take three groups of mice’s eyeballs. Use polyformaldehyde to fix them for 1 h, cut off the complete cornea of the mouse, preserve the limbic tissue, and seal the cornea using bovine serum albumin for 12 h. The expression level of VEGF in the corneal tissue in three groups of mice was detected by enzyme-linked immunoadsorption experiment. The antigen was dissolved by the 50 mM carbonate buffer at the concentration of 10–20 μg/mL, and 100 μL per hole was added to the 96 holes of enzyme marker and the overnight preservation was conducted at 4°C. The following day, the coating solution was removed and washed three times with phosphate-buffered saline with Tween 20 (PBST). Each hole was added with 1% 150 μL bovine serum albumin (BSA) and get sealed for 1 h at 37°C. After that, PBST was used for washing three times, the 100 μL serum of the different ratio was added to each hole, and the control samples were added and incubated at 37°C for 2 h. PBST was used for washing five times, 100 μL of horseradish peroxidase (HRP) marker was used for diluting the double reactance, and the samples were incubated at 37°C for 1 h. The A405 absorption value on the enzyme marker was read after using the color developer for 20 min.

**The detection of c-Fos, c-Jun, ERK1/2, and p-ERK1/2 proteins of corneal tissues in three groups of mice**

Western blot was used to detect c-Fos, c-Jun, ERK1/2, and p-ERK1/2 proteins in mouse corneal tissues. The collected specimens were washed more than three times with PBS buffers. After washing the specimens, the buffers were separated, IP cell lysis solution was added, and the full protein was extracted by cracking for 35 min, and the protein concentration was determined with bicinchoninic acid assay (BCA). Take 20 μg/hole protein, conduct electrophoresis by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, and add the appropriate concentration of SDS-PAGE protein buffer for 15 min. Conduct electrophoresis for 10 min at 100 V. After completing, the electric transition film was soaked into 10% milk and sealed on the shaker for 1.5 h at 37°C. In combination with one resistance, tris-buffered saline with Tween 20 (TBST; by the ratio of 1:1000) was added to the specimens to dilute an anti-β-actin (internal reference) and incubated overnight at 4°C. The next day, clean them with TBST buffer, combined with the double reactance to incubate for 1 h at room temperature, and clean them repeatedly with TBST buffer. Finally, the contrast agent was added to immerse it into the substrate solution for the color development. The gray value of the targeted protein was analyzed through the quantitative reagent kit of BCA protein. The relative expression of the target protein = the gray value of the target protein strip/the gray value of the inner parameter strip.

**Statistical processing**

SPSS19.0 statistical software package was adopted for statistical analysis. The measurement data are described by the mean ± standard deviation (x±SD), the F value is used to test for the comparison among the three groups, the independent
sample t-test is used to test for the comparison among the two groups, and the counting data are described by the number of cases and the percentage, in which $P < 0.05$ is statistically significant.

**Results**

**Eye morphology observation of three groups of mice**

As shown in Figure 1, no angiogenesis was found in the normal group of mice. The basic corneal edema of the mice in the alkali burn group showed the dropsy and had a large number of inflammatory cells and produced a large number of angiogenesis. Inflammatory cell infiltration and the number of new blood vessels of the mice in the inhibitor group decreased significantly.

**Comparison of mice CNV area after modeling 7 days**

As shown in Figure 2, the neovascularization area and length of mice in the alkali burn group were significantly higher than those in the normal group and the inhibitor group, with a statistical difference ($P < 0.05$), and the neovascularization area and length of mice in the inhibitor group were higher than those in the normal group with a statistical difference ($P < 0.05$).

**Histopathological observation of three groups of mice**

As shown in Figure 3, the pathological tissues of three groups of mice were observed. There was no angiogenesis, and the corneal endothelium was intact in the normal group. The formation of CNV and the dense distribution were obvious in the mice of the alkali burn group. The number of CNV in the inhibitor group was significantly lower than that in the alkali burn group, and the neovascularization was sparse and the lumen was small.

**Comparison of expression levels of VEGF in corneal tissue of three groups of mice**

As shown in Figure 4, the expression levels of VEGF in alkali burn group were significantly
higher in corneal tissues than those in normal and inhibitor groups, and the expression level of VEGF of corneal tissue in inhibitor group was higher than that in normal mice with a statistical difference \((P < 0.05)\).

Comparison of relative expression of c-Fos and c-Jun proteins in corneal tissue of three groups of mice

As shown in Figure 5, the relative expression of c-Fos and c-Jun proteins in corneal tissue of the alkali burn group was significantly higher than that in the normal group and the inhibitor group, and the relative expression of c-Fos and c-Jun proteins in the corneal tissue of the inhibitor group was higher than that of the normal group with a statistical difference \((P < 0.05)\).

Comparison of relative expression of ERK pathway protein in corneal tissue of three groups of mice

As shown in Figure 6, the relative expression of ERK1/2 and p-ERK1/2 proteins in the corneal tissue of the alkali burn group was significantly higher than that of the normal group and the inhibitor group. The relative expression of ERK1/2 and p-ERK1/2 proteins in corneal tissues of mice in the inhibitor group was higher than that of mice in the normal group, with a statistically significant difference \((P < 0.05)\).

Discussion

The treatment of CNV not only promotes the degradation of neovascularization but also inhibits the formation of neovascularization. The formation of neovascularization is characterized by the local release of angiogenesis promotion factors and the activation of small venous endothelial cells near the cornea. Thus, endothelial cells produce fibrinogen activators and stimulating blood vessels, which lead to the occurrence of angiogenesis.\(^6,7\) In this research experiment, the model of CNV induced by alkali in mice was established. ERK inhibitors were used to interfere and investigate the effects and the mechanism of ERK inhibitors on neovascularization.

ERK is a member of the filament-based activated protein kinase and is an important component of the ERK signal pathway. After the ERK is activated, it will be converted to phosphorylated ERK, that is, p-ERK. The p-ERK can mediate the transmission of signals from cell plasma to cell nuclei, regulate the physiological effects such as cell growth and division, and participate in the occurrence and development of various diseases.\(^8,9\) Studies have shown\(^10\)
that in patients with eye diseases, the expression of ERK pathway protein was abnormal. The abnormal expression of extracellular protein kinase was shown in corneal tissue of patients’ CNV.\textsuperscript{11} Therefore, in this study, ERK inhibitors were used to investigate the effects and the mechanism of CNV in mice. There are a large number of clinical studies, which show\textsuperscript{12,13} that the use of different drug interventions can effectively inhibit the growth of CNV, which is consistent with the research in this article. In this study, the area of CNV was detected in three groups of mice. The results showed that the CNV area of mice interfered with ERK inhibitors was significantly lower than that of mice without ERK inhibitors, indicating that ERK inhibitors can effectively inhibit the formation of CNV. ERK pathway proteins include ERK1/2 and p-ERK1/2, which showed the high expression in corneal tissue of patients with CNV.\textsuperscript{14,15} In this study, ERK inhibitor intervention was used to detect ERK1/2 and p-ERK1/2 proteins in corneal tissue of mice. The results showed that the expression of ERK1/2 and p-ERK1/2 proteins in corneal tissues of alkali burn mice interfered with ERK inhibitor was significantly lower than that of alkali burn mice without ERK inhibitor, indicating that ERK inhibitors inhibit the abnormal expression of ERK1/2 and p-ERK1/2 proteins in corneal tissues by acting on ERK protein pathways, thereby inhibiting the growth of CNV. Studies have shown that\textsuperscript{16,17} the use of ERK inhibitors in animals of CNV model can effectively inhibit the growth of neovascularization, promote the degradation of neovascularization, and improve the symptoms of neovascularization of the cornea, thereby improve visual function.

VEGF is an endothelium-specific factor that can effectively promote mitosis, also the formation of angiogenesis, and maintain the integrity and normal state.\textsuperscript{18,19} Studies have shown\textsuperscript{20} that the expression of VEGF in CNV was significantly increased and is a key regulator of major CNV. In this study, the expression level of VEGF in corneal tissue of three groups of mice was detected. The results showed that the expression level of VEGF in corneal tissue of mice in alkali burn group was significantly higher than that of normal mice. ERK inhibitor intervention was carried out in mice of inhibitor group. The results showed that the expression level of VEGF in corneal tissue of mice with inhibitors decreased significantly, indicating that ERK inhibitors could effectively reduce the
expression of VEGF in mouse corneal tissue and inhibit the formation of CNV. A large number of clinical studies have shown\textsuperscript{21,22} that the expression level of VEGF in CNV is significantly higher than that of normal body. Through the intervention of inhibitors, the expression level of VEGF can be effectively improved, thereby inhibiting the occurrence of CNV, which is consistent with the results in this study.

Studies have shown\textsuperscript{23,24} that c-Fos, c-Jun, and ERK are the members of filament-based activated protein kinase involved in the inflammatory reaction of the body and show the lower expression level of the normal body, mainly regulating the growth and differentiation of various cells. c-Fos is expressed in low normal cells and is mainly regulated by a variety of inflammatory cells, inflammatory media, and inflammatory factors. Under the stimulation of inflammatory reactions, the media and cytokines of the body are activated.\textsuperscript{25} Studies have shown\textsuperscript{26} that c-Jun plays an important role in regulating pathways and is mainly involved in cell metabolism and growth. In this study experiment, ERK inhibitor intervention was performed on alkali burned mice to detect the expression of c-Fos and c-Jun in the corneal tissue of mice. The results showed that the expression levels of c-Fos and c-Jun in the corneal tissues of mice with ERK inhibitors were significantly lower than that of mice without ERK inhibitors, indicating that ERK inhibitors acting on ERK pathways regulated the abnormal expression of c-Fos and c-Jun, which inhibits the growth of new angiogenesis.

In summary, ERK inhibitors inhibit the expression of VEGF, c-Fos, and c-Jun proteins by acting on the ERK signal pathway, thereby inhibiting the formation of CNV.

**Declaration of conflicting interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) received no financial support for the research, authorship, and/or publication of this article.

**ORCID iD**
Manhua Xu https://orcid.org/0000-0002-4949-5214

**References**

1. Oguido APMT, Hohmann MSN, Pinho-Ribeiro FA et al. (2017) Naringenin eye drops inhibit corneal neovascularization by anti-inflammatory and antioxidant mechanisms. *Investigative Ophthalmology & Visual Science* 58(13): 5764–5776.
2. Zong R, Zhou T, Lin Z et al. (2016) Down-regulation of MicroRNA-184 is associated with corneal neovascularization. *Investigative Ophthalmology & Visual Science* 57(3): 1398–1407.
3. Aksoy S (2019) Treatment of corneal neovascularization with topical aflibercept in a case of exposure keratopathy following cerebellar astrocytoma surgery. *Indian Journal of Ophthalmology* 67(1): 145–147.
4. Ding XY, Gu RP, Tang WY et al. (2018) Effect of phosphorylated-extracellular regulated kinase 1/2 inhibitor on retina from light-induced photoreceptor degeneration. *Chinese Medical Journal* 131(23): 2836–2843.
5. Mei ZB, Duan CY, Li CB et al. (2016) Prognostic role of tumor PIK3CA mutation in colorectal cancer: A systematic review and meta-analysis. *Annals of Oncology: Official Journal of the European Society for Medical* 27(10): 1836–1848.
6. Nominato LF, Dias AC, Dias LC et al. (2018) Prevention of corneal neovascularization by adenovirus encoding human vascular endothelial growth factor soluble receptor (s-VEGFR1) in lacrimal gland. *Investigative Ophthalmology & Visual Science* 59(15): 6036–6044.
7. Filippi L, de Libero C, Zamma Gallarati B et al. (2018) Propranolol eye drops in patients with corneal neovascularization. *Medicine (Baltimore)* 97(45): e13002.
8. Miao R, Lu Y, Xing X et al. (2016) Regulator of G-protein signaling 10 negatively regulates cardiac remodeling by blocking mitogen-activated protein kinase-extracellular signal-regulated protein kinase 1/2 signaling. *Hypertension* 67(1): 86–98.
9. Hiratsuka T, Sano T, Kato H et al. (2017) Live imaging of extracellular signal-regulated kinase and protein kinase A activities during thrombus formation in mice expressing biosensors based on Förster resonance energy transfer. *Journal of Thrombosis and Haemostasis* 15(7): 1487–1499.
10. Pan J, Zhang D, Zhang J et al. (2018) LncRNA RMRP silence curbs neonatal neuroblastoma progression by regulating microRNA-206/tachykinin-1 receptor axis via inactivating extracellular signal-regulated kinases. *Cancer Biology & Therapy* 20(5): 653–665.
11. Sato T, Arakawa M, Tashima Y et al. (2018) Statins reduce thoracic aortic aneurysm growth in Marfan syndrome mice via inhibition of the Ras-induced ERK (extracellular signal-regulated kinase) signaling pathway. *Journal of the American Heart Association* 7(21): e008543.
12. Xu X, Liu T and Li H (2018) Effect of collagen cross-linking on alkali burn-induced corneal neovascularization in rabbits. *Journal of Ophthalmology* 2018: 7325483.

13. Bignami F, Lorusso A, Rama P et al. (2017) Growth inhibition of formed corneal neovascularization following Fosaprepitant treatment. *Acta Ophthalmologica* 95(7): e641–e648.

14. Liu CH, Lan CT, Chen LY et al. (2018) Phosphorylation of extracellular signal-regulated kinase 1/2 in subepidermal nerve fibers mediates hyperalgesia following diabetic peripheral neuropathy. *Neurotoxicology* 71: 60–74.

15. Gaspar RC, Muñoz VR, Kuga GK et al. (2019) Acute physical exercise increases leptin-induced hypothalamic extracellular signal-regulated kinase1/2 phosphorylation and thermogenesis of obese mice. *Journal of Cellular Biochemistry* 120(1): 697–704.

16. Duan CY, Zhang J, Wu HL et al. (2017) Regulatory mechanisms, prophylaxis and treatment of vascular leakage following severe trauma and shock. *Military Medical Research* 4: 11.

17. Cho WK, Kang S, Choi H et al. (2015) Topically administered gold nanoparticles inhibit experimental corneal neovascularization in mice. *Cornea* 34(4): 456–459.

18. Senturk B, Cubuk MO, Ozmen MC et al. (2016) Inhibition of VEGF mediated corneal neovascularization by anti-angiogenic peptide nanofibers. *Biomaterials* 107: 124–132.

19. García-Caballero M, Blacher S, Paupert J et al. (2016) Novel application assigned to toluquinol: Inhibition of lymphangiogenesis by interfering with VEGF-C/VEGFR-3 signalling pathway. *British Journal of Pharmacology* 173(12): 1966–1987.

20. Chang JH, Garg NK, Lunde E et al. (2012) Corneal neovascularization: An anti-VEGF therapy review. *Survey of Ophthalmology* 57(5): 415–429.

21. Voiculescu OB, Voinea LM and Alexandrescu C (2015) Corneal neovascularization and biological therapy. *Journal of Medicine and Life* 8(4): 444–448.

22. Shen M, Zhou XZ, Ye L et al. (2018) Xanthatin inhibits corneal neovascularization by inhibiting the VEGFR2-mediated STAT3/PI3K/Akt signaling pathway. *International Journal of Molecular Medicine* 42(2): 769–778.

23. Lesch A, Rössler OG and Thiel G (2017) Extracellular signal-regulated protein kinase, c-Jun N-terminal protein kinase, and calcineurin regulate transient receptor potential M3 (TRPM3) induced activation of AP-1. *Journal of Cellular Biochemistry* 118(8): 2409–2419.

24. Xiao X, Song D, Cheng Y et al. (2018) Biogenic nanoselenium particles activate Nrf2-ARE pathway by phosphorylating p38, ERK1/2, and AKT on IPEC-J2 cells. *Journal of Cellular Physiology* 234(7): 11227–11234.

25. Yamazaki Y, Arita K, Harada S et al. (2018) Activation of c-Jun N-terminal kinase and p38 after cerebral ischemia upregulates cerebral sodium-glucose transporter type 1. *Journal of Pharmacological Sciences* 138(4): 240–246.

26. Bi C, Cai Q, Shan Y et al. (2018) Sevoflurane induces neurotoxicity in the developing rat hippocampus by upregulating connexin 43 via the JNK/c-Jun/AP-1 pathway. *Biomedicine & Pharmacotherapy* 108: 1469–1476.