Epigallocatechin gallate inhibits corneal neovascularization in ratalkaline burn model

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Abstract: To evaluate the effectiveness of epigallocatechin gallate (EGCG) in inhibiting corneal neovascularization in rat alkaline burn model. Corneal neovascularization model was induced by sodium hydroxide alkaline burn injury in SD rats. Rats were randomly divided into two groups and were given intraperitoneal injection with EGCG or PBS per day for up to 14 days respectively. Corneal inflammation and neovascularization area were assessed on days3, 7, and 14 after cauterization with digital photographs. Vascular endothelial growth factor (VEGF) and pigment epithelium derived factor (PEDF) mRNA levels were measured by reverse transcription-polymerase chain reaction (qRT-PCR). The nuclear-transfactor-Kb (NF-κB) subunit P65 protein was assayed by immunohistochemistry. The differences of corneal inflammation scores between two groups were significant. The area of CNV between two groups had no significant difference on day3 but have significant difference on days 7 and 14.The *PDEF* mRNA expression in EGCG group was significantly higher and the expression of VEGF mRNA was lower than those in PBS group. The results of immunohistochemistry showed from day 7, expression of NF-κB P65protein was suppressed considerably in EGCG group. This study demonstrates that EGCG inhibits corneal neovascularization in a rat model induced by alkali burn.

Keywords: EGCG; corneal neovascularization; VEGF; PEDF; NF-κB.

INTRODUCTION

Corneal alkaline burn is a common traumatic ocular disease encountered worldwide particularly in developing countries. It represents a complicated pathology that may permanently affect visual function and may even lead to blindness. One of the most important pathological factorsin loss of vision is corneal neovascularization (CNV). At the same time, corneal NV is the top high-risk factor in corneal allograft transplantation (Cursiefen *et al*., 2001). Therefore, reducing CNV as early as possible is essential to the treatment.

The mechanism of CNV is known to be the imbalance between angiogenic and anti-angiogenic factors in cornea (Han, *et al*., 2015). Vascular endothelial growth factor (VEGF) is the most essential angiogenic factor in the cornea tissue (Ozdemir, *et al*., 2014). While the pigment epithelium derived factor (PEDF) is a valid anti-angiogenic factor in ocular tissue (Mirochnik, *et al*., 2009). Nuclear factor kappa B (NF-κB) pathway plays an important role for majority of cytokines signaling on ligand binding to cell surface receptors. NF-κB is the principal transcription factor that controls various biological processes, including corneal wound healing, inflammation, angiogenesis, apoptosis and the stress response (Cildir, *et al*., 2016). Studies have shown that NF-κB positively adjusts the expression of VEGF mRNA and protein and VEGF can enhance the NF-κB combining ability in a positive feedback way (Watari *et al*., 2012; Kubota, *et al*., 2011).

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Epigallocatechin gallate (EGCG), a polyphenolic compound plentiful in green tea, has been proved to be effective in anti-oxidative, anti-inflammatory, and anti-atherogenic (Hossain, *et al*., 2014). Recently, an in vivo study utilizing mice verified that NF-κB may be involved in EGCG lowing vascular inflammation (Liu, *et al*., 2016). In this study, we used rat corneal alkaline burn model to evaluate the effectiveness of EGCG inhibiting CNV and to elucidate its possible mechanism.

MATERIALS AND METHODS

Animals

Sixty male Sprague Dawley (SD) rats (180-200 g) were purchased from Zhejiang University Animal Laboratory center, Hangzhou, China. This study was performed in conformity to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Experimental Animal Committee of Zhejiang University.

Corneal alkaline burn model and EGCG treatment

Rats were randomly divided into 2 groups: (1) EGCG group: Rats received intraperitoneal injection with EGCG (50mg/Kg) per day for up to 14 days; (2)Contra group: Rats were intraperitoneal injection with 0.01mol/L PBS per day for up to 14 days. The rats were anesthetized with an intraperitoneal injection of 10ml/kg 4% chloral hydrateand the right eyes received topically a drop of oxybuprocaine hydrochloride. After wards, a filter paper disc (4.0 mm in diameter) soaked with 1moL/LNaOH was placed on the center of the corneal surface for 1min.After

alkali burns, the ocular surface was irrigated with 0.9% saline for 30s immediately and then the rats were injected intraperitoneally with EGCG or PBS. Antibiotic drops and atropine ointment were used daily. All eyes were observed to assess the baseline of CNV, inflammation and fluorescein staining under a slit lamp microscope. Ten rats in each group underwent cervical spine dislocation to death on the third day, seventh day and fourteenth day, respectively. Cornea was split into two parts. Half was prepared for HE staining and Immunohistochemistry after fixed with 10% formalin, the other half was saved in -80^{o}C refrigerator was used for qRT-PCR.

Score of corneal inflammation

According to the following assessment, the index of inflammatory values were scored: central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3); ciliary hyperemia (absent, 0; present but extending less than 1 mm, 1; hyperemia extending between1 and 2 mm, 2; present and extending more than 2 mm, 3).The final inflammatory index result was obtained by summing the scores of the different parameters (Han, *et al*., 2015).

Corneal NV evaluation

Images were obtained under a slit lamp microscope. Vessel length was determined by measuring the perpendicular distances between the limbus and vessel tips. The CNV area (A) was calculated using the following equation: A=C/12x3. 1416x[r2-(r-l)2] (A is the area, C is time, l is the radius to the border of the vessel, and r is the radius of the cornea)(Zhang, *et al*., 2006).

Immunohistochemistry

Rat cornea samples were fixed overnight in 4% formaldehyde in PBS, followed by dehydration in a series of ascending alcohol and paraffin embedding. The paraffin sections were removed paraffin and then immersed in 1.5% methanol hydrogen peroxide blocking for 10 minutes. Then the sections were put into a preheated PH8.0 EGTA boiling for 20 minutes after distilled water rinsing. Sections were then incubated with the primary antibody to NF-kB P65 (1:400 dilution; Abcam Biotechnology, Inc) at 37^{o}C# for 60 minutes according to the manufacturer's protocols. After incubated with the secondary antibody (PV8000, Zhongshan Jinqiao Company) and washed with PBS, the sections were counterstained with hematoxylin, dehydrated and mounted for light microscopy observations. For control sections the primary antibody was omitted.

qRT-PCR for VEGF and PEDFmRNA

The corneas were cut into small pieces and homogenized in TRIzol (Invitrogen) on ice using a ULTRA-TURRAX digital homogenizer (IKA, Staufen, Germany).Total RNA was extracted using Trizol reagent in accordance with standard procedures. RNA samples were treated with the First Strand cDNA Synthesis Kit Rever TraAce-α-kit (Toyobo Company) according to the manufacturer's instructions. Total RNA (1μg) from each sample was reverse transcribed into cDNA and real-time PCR was performed using the Thunderbird SYBR qPCR Mix kit (Toyobo Company) in accordance with the manufacturer's instructions. Relative RNA expression was calculated using the $2^{(-\Delta\Delta CT)}$ method. The primer sequences for *VEGF* gene were 5'-TCAAGCCGTCCT GTGTGCC-3'(forward) and 5'-AACAAATGCTTTCTC CGCTCTG-3'(reverse). The primer sequences for *PEDF* gene were 5'-CACTGGCAACCCTCGCATAG-3' (forward) and 5'-CAAACTTGGTTGCCCACTGC-3' (reverse). The actin gene, as an internal control, was assessed with the primers 5'-CTGTGGCTACTGGTG CTGACG-3' (forward) and 5'-CCTCCTTGGCGTAG TAGTCGG-3' (reverse).

RESULTS

EGCG suppressed corneal inflammation and NV

Corneal inflammation and NV were observed simultaneously. Both groups of rats presented limbus vessels congestion, epithelial edema and brush-shape CNV formation on day 3. Butin PBS group the corneal edema was more serious, of ten characterized by bullous keratopathy. Then, the CNV developed from the limbus to the center of the cornea by day 7. The CNVs were shorter and thinner in EGCG group than those in PBS group. Corneal stromal opacification was observed more frequently and severe CNVs in the PBS group at days 14 as compared with EGCG treated group (fig. 1).

The corneal inflammation scores on day 3 were 6.5±2.2 and 5.7±0.6 in PBS group and EGCG group with significant difference ($P<0.05$). On day 7 and day 14 the scores in EGCG group were5.6±0.5 and 4.4±0.7 and in PBS group those were 8.5±0.7 and 7.4±0.7.The differences on both day were significant ($P<0.01$)(fig. 2A). The area of CNV between two groups had no significant difference on day3 scored with 7.87±1.50 and 7.25±2.56 in PBS and EGCG group ($P>0.5$). On day 7 the CNV area increased to20.16±3.14 and 16.24±2.40 in PBS and EGCG group respectively with a significant difference($P<0.05$).Till to the day 14,the CNV area in EGCG group decreased to13.85±2.51 sharply, while in PBS group the CNV area slightly decreased to18.87±3.53.The difference was significant obviously ($P<0.01$) (fig. 2B).

EGCG enhances the anti-angiogenic factor PDEF and inhibits angiogenic factor VEGF

Realtime PCR was performed to investigate the effects of EGCG treatment on the *PDEF* and *VEGF*mRNA expression. The *PDEF* mRNA expression induced by EGCG treatment reached its highest level on third day

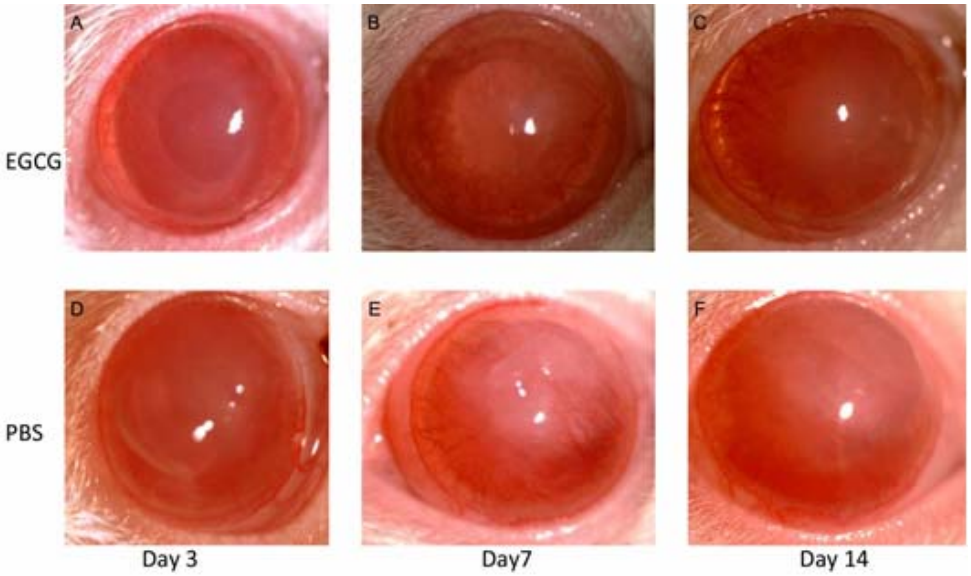

Fig. 1: Macroscopic observation showed limbus vessels congestion, brush-shape CNV formation in both groups, and light epithelial edema in EGCG group (A) and bullous keratopathy in PBS group (D) at day 3 after alkaline burning. The CNVs were shorter and thinner in EGCG group (B) than in PBS group (E) by day 7. The corneal central opacity and CNVs were less severe in the EGCG group(C) than in the PBS group (F) on day 14.

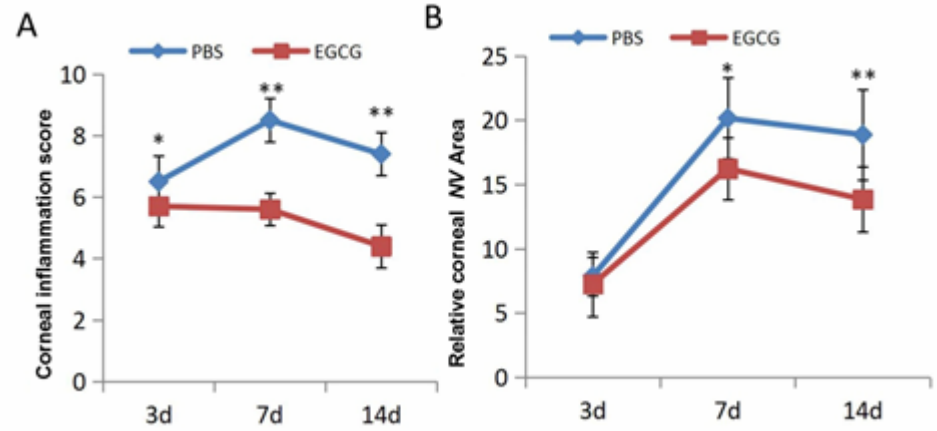

Fig. 2: The differences of corneal inflammation scores between two groups were significant (fig. 2A). The area of CNVs had no significant difference on day3 but had significant difference on day 7 and 14 (fig. 2B) (*P<0.05, **P<0.01).

and decreased gradually. No matter observation on which day, the *PDEF* mRNA expression in EGCG group was significantly higher than those in PBS group (P<0.01) (fig. 3A). The expression of *VEGF* mRNA in both group increased after cauterization. In PBS group it reached the highest level on 7 day and in EGCG group decreased to a lower level until the fourteenth day. There was significant difference between the two groups (fig. 3B).

EGCG treatment suppresses the expression of p65 subunit of NF-κB

In the normal cornea, NF-κB P65 presents weakly positive expression in epithelial cells. On the third day after cauterization, the results of immunohistochemistry showed the expression were positive strongly in both groups. Till to the seventh day, expression of NF-κB P65was weakly positive in the corneal matrix but still strongly in epithelium in EGCG group. While in PBS group, expression in epithelium and matrix were still positive strongly. The expression was obviously weaker in EGCG group than in PBS group on day 14 regardless in epithelium or in matrix (fig. 4).

DISCUSSION

It is widely known that alkali burns causes acute inflammation and neovascularization in the cornea. CNV is not only regarded as the most important factor that leads to the missing of transparency but also increases the likelihood of rejection reaction after corneal transplantation. Glucocorticoid is the most common drug to inhibit the CNV, but it cannot be used for long time because of ocular side effects. EGCG is a natural anti-inflammatory agent that has been reported to resist

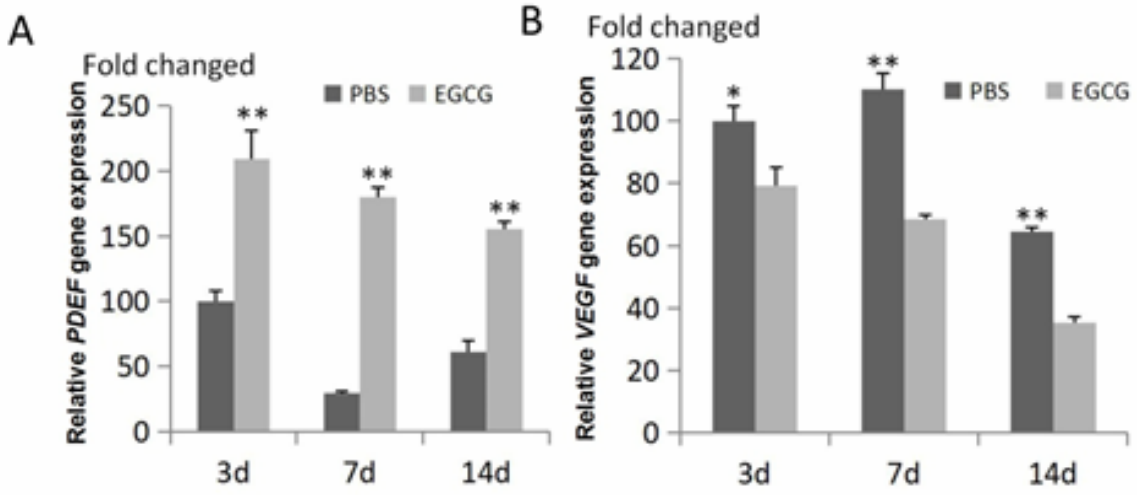

Fig. 3: Compared with PBS group, the relative *PDEF* mRNA expression was significantly higher (fig. 3A) and the expression of *VEGF* mRNA was significantly lower (fig. 3B) in EGCG group (*P<0.05,**P<0.01).

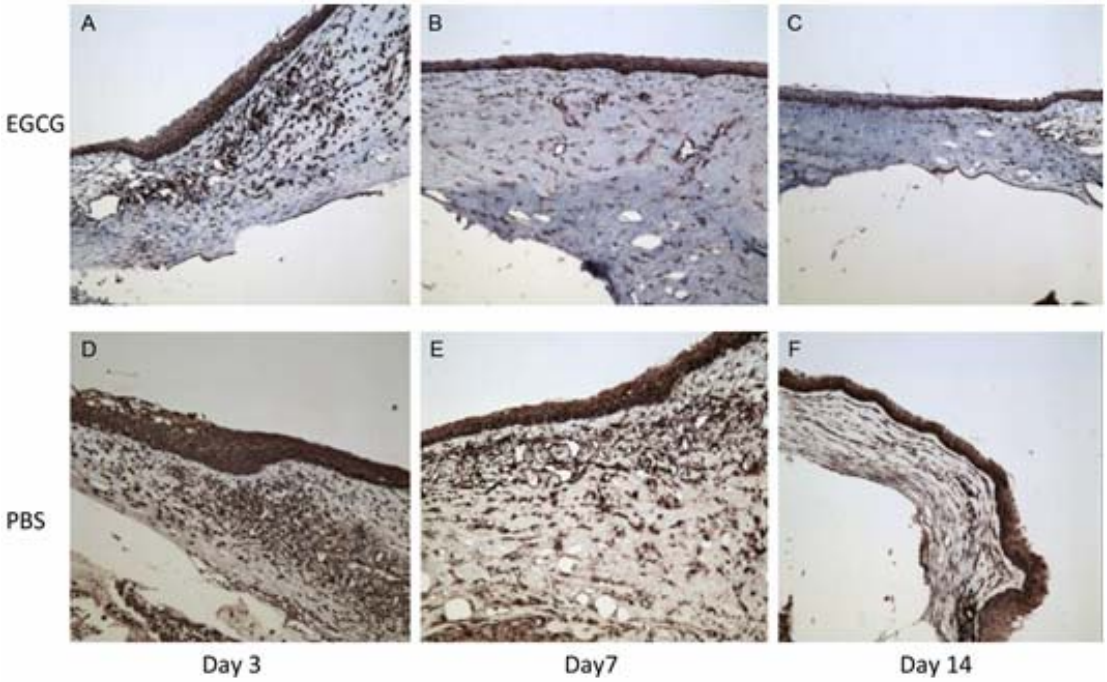

Fig. 4: The results of immunohistochemistry showed the expression of NF-κB P65were positive strongly in both groups on day 3 after cauterization (A and D). On day 7, the expression was weakly positive in the corneal matrix but still strongly in epithelium in EGCG group (B). In PBS group, expression was still positive strongly in epithelium and matrix (E). The expression was obviously weaker in EGCG group than in PBS group on day 14 regardless in epithelium or in matrix (C and F).

various inflammatory processes. In this study, we showed that EGCG significantly inhibited the expression of VEGF and increased the expression of PEDF. Additionally, we observed significant changes of NF-κB p65protein between EGCG and PBS group. EGCG is confirmed to have inhibitory effects on CNV. We proposed the inhibitory effects of EGCG on CNV were attributed to the regulation of angiogenic and anti-angiogenic factors and inhibition of the NF-κB pathway.

The regulation of corneal neovascularization is a complex process controlled by stimulation and inhibition factors. Studies have found that VEGF played a key mediating role in the process of neovascularization (Sari, *et al*., 2015). As an important member of this family, VEGF-A promotes vascular endothelial cell proliferation, migration and tube formation during the process of pathologic hemangiogenesis (Rodrigues *et al*., 2009). A number of studies have shown that anti-VEGF is an effective

treatment for CNV (Ozdemir, *et al*., 2012). PEDF is a most available inhibitor of VEGF-induced neovascularization, which have also been showed in various animal models. PEDF reduces the activity of VEGF by the way of binding competitively to the pro-angiogenic VEGF-R2 and inhibiting the phosphorylation of VEGF-R1 (Zhang, *et al*., 2006; Cai *et al*., 2006). According to the pathogenesis of CNV, the treatment has achieved rapid development both at home and abroad in recent year (Kuo, *et al*., 2013). Ozdemir showed whether subconjunctival injection or topical administrations of bevacizumab, could reduce inflammation, decrease the activity of fibroblasts and inhibit the new vascularization of cornea in a rat model of alkaline burn (Ozdemir, *et al*., 2014). Kuerten D reported that corneal neovascularization induced by alkali can be inhibited after PEDF-transfected pigment epithelial cells were implanted sub conjunctivally in rabbits (David, *et al*., 2015). EGCG is the primary polyphenol ingredient of green tea, which has antioxidant, anti-inflammatory, antiviral and anticancer effects (Karthikeyan, *et al*., 2017). In addition, experimental evidence suggests that drinking green tea from mice significantly inhibits angiogenesis. There are very few researches about applying EGCG to treat the CNV. Lee HS demonstrated that EGCG effectively limited corneal neovascularization induced byalkaline burnby repressing the level of MMP-9 and platelet endothelial cell adhesion molecule (Lee, *et al*., 2014). Moyle CW showed that in human umbilical vein endothelial cells the EGCG potently inhibit VEGF-induced VEGF receptor 2 by interacting with VEGF directly (Moyle, *et al*., 2015). Our studies confirmed that intraperitoneal injection of EGCG enhanced the anti-angiogenic factor PDEF and inhibited angiogenic factor VEGF.

Meanwhile, we were trying to study how the EGCG regulated these factors. Among the various pathways involved in controlling angiogenic and anti-angiogenic factors regulation, the NF-κB signaling pathway is recognized and studied (Babu *et al*., 2012; Poon, *et al*., 2017). In some ocular surface disorders, such as chemical injury, pterygium and corneal graft rejection, NF-κB migrates to the nucleus after NF-κB dissociation from IκB-α, where it combines with the promoters of NF-κB-regulated genes and activates gene transcription (Li, *et al*., 2002; Lan, *et al*., 2012). Kim H reported that NF-κB can facilitate the expression of matrix metalloproteinase, MMP9 and MMP2, thus leading to the formation of CNV (Kim, *et al*., 2000). In our studies, the level of NF-κB p65 peaked 3 days after cautery in both group, but its level decreased in EGCG treatment group.

In summary, our studies showed EGCG inhibited CNV in rat alkaline burn model. It enhanced the anti-angiogenic factor PDEF and inhibited angiogenic factor VEGF by controlling NF-κB activity. In the following work, we move on to research other possible mechanisms and signal pathways of EGCG inhibiting CNV and demonstration the ocular surface toxicity and effectiveness when EGCG was used with eye drops.

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