Diospyros kaki Extract Inhibits Alkali Burn-Induced Corneal Neovascularization

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ABSTRACT The purpose of this study was to evaluate the effect of ethanol extract of *Diospyros kaki* (EEDK) leaves on corneal neovascularization (CoNV) in rats. One week after the alkali burns in the corneas, the CoNV area coverage in the CoNV-positive control group, 100 mg/kg EEDK group, and 200 mg/kg EEDK group was $43.3\% \pm 5.5\%$, $337.7\% \pm 2.5\%$, and $27.2\% \pm 4.3\%$, respectively. The areas of CoNV in the EEDK-treated groups were significantly different from those of the CoNV group. EEDK significantly attenuated the upregulation of vascular endothelial growth factor, fibroblast growth factor, interleukin-6, and matrix metalloproteinase-2 (MMP-2) protein levels. Orally administrated *D. kaki* inhibited CoNV development in rats.

KEY WORDS: • alkali burn • angiogenic factors • corneal neovascularization • *Diospyros kaki* • eye diseases

CORNEAL NEOVASCULARIZATION (CoNV) is a vision-threatening condition that is usually caused by inflammatory, infectious, or traumatic events at the corneal surface.¹ Although great efforts have been expended to reduce and prevent CoNV, no specific treatment against CoNV is currently available. Due to remarkable developments made in angiogenesis research in recent years, novel specific antiangiogenic drugs have been used against cancer and neovascular diseases in the retina and choroid.²

The process of new blood vessel formation requires proliferation, migration, and capillary tube formation by endothelial cells. The infiltration of inflammatory cells and the release of several angiogenesis-related mediators contribute to CoNV.³

Diospyros kaki (Asian persimmon) has been cultivated throughout Eastern Asia for centuries and is traditionally used in herbal medicine owing to its beneficial properties, which include antiatherosclerotic, antidiabetic, anti-inflammatory, and antineurodegenerative effects.^{4–7} The leaves of *D. kaki* contain an abundance of flavonoids, the most common group of polyphenolic compounds, which are responsible for the beneficial effects of *D. kaki*.⁸ Recently, several studies have shown that dietary flavonoids act as anti-inflammatory phytochemicals that inhibit proinflammatory and proangio-

genic cytokines, such as vascular endothelial growth factor (VEGF), interleukin-1 β (IL-1 β), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-9 (MMP-9), during CoNV⁹ and effectively ameliorate experimentally induced inflammation in models of dry eyes.¹⁰

This study aimed to investigate the protective effects of the ethanol extract of *D. kaki* (EEDK) leaves on alkali burn-induced CoNV in rats.

The leaves of *D. kaki* were collected from Gangneung in the Gangwon Province of Korea in June 2013. A voucher specimen (D-521) was deposited at the KIST Gangneung Institute. The dried leaves of *D. kaki* (800 g) were extracted twice with ethanol and filtered. The combined filtrate was concentrated and dried by rotary evaporation to obtain 23.8 g of EEDK.

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines established by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Alkali burn was inflicted by the 10-sec application of a 3-mm-diameter filter paper soaked in 1 mol/L NaOH. After producing alkali burns, 24 rats were divided into three groups, which received either 100 mg/kg (1) or 200 mg/kg (2) EEDK daily by gavage and a positive control (3; CoNV group).

After 7 days, all rats were examined using biomicroscopy, and anterior segment photos were taken by a digital camera mounted on a TRC-50IX retina camera (Nikon) at the same focus setting ($\times 16$). Photographs were examined using an image analyzing program (Image J 1.40g; National

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FIG. 1. Comparison of the corneal neovascularization (CoNV) area on slit lamp microscopic photographs. **(A)** Percentage of the vascularized area/total cornea area. Representative photographs after chemical injury: **(B)** CoNV, **(C)** 100 mg/kg ethanol extract of *Diospyros kaki* (EEDK), and **(D)** 200 mg/kg EEDK. Experimental values are expressed as mean $\pm$ standard error of the mean from three independent experiments. Mann–Whitney *U*-test, * $P < .05$, *** $P < .001$.

FIG. 2. Western blot analysis of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin-6 (IL-6), and matrix metalloproteinase-2 (MMP-2). Each bar represents the fold-induction of cytokine protein levels relative to the control and an asterisk denotes significant difference from control (* $P < .05$). Experimental values are expressed as mean $\pm$ SEM from three independent experiments. Mann–Whitney *U*-test, * $P < .05$.

Institutes of Health). All corneas and neovascularization therein were outlined with reference to the corneal limbus. To determine the percentage of CoNV, the number of pixels showing neovascularization was expressed as a percentage of the total corneal pixel number. The area of CoNV was compared between the three groups.

Experimental values are presented as mean $\pm$ standard deviation and were analyzed by the Kruskal–Wallis and Mann–Whitney tests using the Bonferroni correction. A result of $P < .05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS version 16.0 (IBM Corp.).

The corneal photographs with neovascularization are shown in Figure 1. One week after the chemical burn, the percentage of CoNV area was $43.3\% \pm 5.5\%$, $337.7\% \pm 2.5\%$, and $27.2\% \pm 4.3\%$ in the CoNV group, 100 mg/kg EEDK group, and 200 mg/kg EEDK group, respectively (Fig. 1). The percentage of CoNV was significantly lower in the groups treated with 100 mg/kg EEDK and 200 mg/kg EEDK than in the CoNV group ($P = .001$, Kruskal–Wallis test). Furthermore, the percentage of CoNV in the 200 mg/kg EEDK group was significantly lower than that in the 100 mg/kg EEDK group ($P = .026$, Mann–Whitney test).

One week after chemical burning of the cornea, the protein expression levels of VEGF, fibroblast growth factor (FGF), interleukin-6 (IL-6), and MMP-2 were determined using western blot analysis. When compared with the negative control group (without chemical burns), the induction of CoNV significantly increased the abundance of angiogenic and inflammatory factors VEGF, FGF, IL-6, and MMP-2. However, EEDK significantly attenuated the upregulation of VEGF, FGF, IL-6, and MMP-2 produced by corneal burning (Fig. 2). The expression levels of these proteins were not significantly lower in the 100 mg/kg EEDK group compared with the CoNV group, with the exception of MMP-2. There were statistically significant differences in the abundance of VEGF, FGF, IL-6, and MMP-2 between the CoNV group and the 200 mg/kg EEDK group. These findings demonstrate that EEDK reduced the expression of proteins involved in CoNV in a dose-dependent manner.

The wound healing process is orchestrated by various signals derived from endogenous soluble factors. IL-1 β , IL-6, and TNF- α are expressed in alkali-burned corneas, and IL-6, in particular, is strongly induced soon after alkali burn injury in mice.¹¹

VEGF has been detected in alkali-burned corneas in mice.¹² MMPs have been implicated in the development of CoNV.¹³ MMPs are zinc-dependent extracellular endoproteinases that disrupt cell-to-cell and cell-to-extracellular matrix connections and facilitate the migration of endothelial cells to areas stimulated by VEGF, ultimately leading to the formation of new vasculature.^{13,14}

These results suggest that EEDK suppressed CoNV at least partly by inhibiting the expression of VEGF, FGF, IL-6, and MMP-2.

It has been reported that *D. kaki* contains high amount of flavonoids, such as quercetin and kaempferol derivatives,

which have shown inhibitory potencies for proangiogenic cytokines such as VEGF, IL-1 β , MMP-2, and MMP-9 during CoNV. Thus, these flavonoids could possibly be the active compounds that exert the antiangiogenic effects of EEDK.^{8,9}

The results presented here suggest that EEDK should be considered a candidate for the treatment of angiogenesis-related ocular diseases, including retinal vascular diseases such as diabetic retinopathy, retinal vein occlusion, and exudative age-related macular degenerations. Future investigations should evaluate the effects of EEDK in such angiogenesis-related ocular conditions.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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