DOXYCYCLINE ENHANCES THE INHIBITORY EFFECTS OF BEVACIZUMAB ON CORNEAL NEOVASCULARIZATION AND PREVENTS ITS SIDE EFFECTS

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PURPOSE. To investigate the combination therapeutic effects of topical doxycycline temperature-sensitive hydrogel (DTSH) and bevacizumab on corneal neovascularization (CNV) and corneal wound healing (CWH) and to explore the underlying mechanisms of doxycycline on CNV and CWH.

METHODS. Rats were treated with a saline solution, topical DTSH (0.1%), topical bevacizumab (2.5 mg/0.1 mL), or a DTSH and bevacizumab combination. For the bFGF-induced CNV model (n = 15/group), the length and area of CNV were measured on day 7. In the alkali burn model (n = 33/group), the length and area of CNV were determined on days 3, 7, 14, and 21 after alkali burn. The activity of matrix metalloproteinase (MMP)-2 and MMP-9 was determined by a fluorogenic peptide substrate. Western blot, real-time PCR, and ELISA were used to analyze the expression of induced nitric oxide synthase (iNOS), VEGF, VEGFRs, MMP-2, MMP-9, and IL-1β.

RESULTS. Combination therapy more effectively inhibited CNV than therapy with topical bevacizumab or DTSH alone. DTSH combined with bevacizumab significantly accelerated delayed CWH caused by topical bevacizumab in the alkali burn model (P = 0.018). Combination therapy showed better inhibitory effects on MMP expression and phosphorylated VEGFR1 and VEGFR2. With DTSH treatment, doxycycline inhibited the activity and expression of MMPs, the expression of VEGF and of phosphorylated VEGFR1 and VEGFR2, and the production of iNOS and IL-1β in local cornea.

CONCLUSIONS. Doxycycline enhances the inhibitory effects of bevacizumab on CNV and prevents its side effects on CWH, possibly by inhibiting the expression and activity of MMPs, the expression of VEGF and of phosphorylated VEGFR1 and VEGFR2, and the production of iNOS and IL-1β.

Corneal neovascularization (CNV), a common sight-threatening complication in a variety of ocular surface disorders, is still a therapeutic challenge for ophthalmologists.

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bined therapeutic effect of topical administration of DTSH and bevacizumab on CNV and CWH and to explore the underlying mechanisms of doxycycline effects on CNV and CWH.

**Materials and Methods**

**Animals**

Female Sprague-Dawley rats (6–8 weeks, 180–200 g) were obtained from the Guangzhou Animal Testing Center. The studies were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University. All procedures involving animal eye studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were performed consistently by the same investigator. The right eyes of the animals were used for experiments, and the left eyes were left intact.

**Preparation of DTSH**

DTSH was prepared as described in our previous study. In brief, doxycycline (Sigma-Aldrich, St. Louis, MO), hydroxypropyl-β-cyclodextrin, poloxamer 407 (Sigma-Aldrich), and poloxamer 188 (Sigma-Aldrich) were mixed at a mass ratio of 1:24:220:35, respectively. The prepared DTSH was sterilized by passage through a 0.2-m filter (Corning, NY) and was stored at 4°C before use.

**bFGF-Induced CNV Model**

For assessment of CNV caused by bFGF in vivo, we used a corneal micropocket assay. In brief, an aliquot of bFGF (R&D Systems, Inc., Minneapolis, MN) was added to 3 μL of 12% (wt/vol) polymer (Hydron; Sigma-Aldrich) in ethanol. The mixture was deposited in 3-μL drops onto an autoclaved 2-mm nonstick sheet (Telfon; Small Parts, Inc., Miami Lakes, FL) using a micropipette. The drops became solid pellets after drying overnight in a desiccator. Each pellet contained approximately 100 ng bFGF. A partial corneal incision was made through the corneal center, and a micropocket was created with a modified iris spatula. The shortest distance between the bottom of the micropocket and the limbus was approximately 1.4 mm. A prepared sterile pellet (Hydrion; Sigma-Aldrich) was placed into the corneal micropocket.

**Alkali Burn Model**

To create a serious alkali burn model, a circular piece of Whatman #5 filter paper (4 mm in diameter) was dropped into 5 μL of 1 M sodium hydroxide and then placed in the center of the right eye of a rat for 60 seconds. The burned area and conjunctival sac were irrigated with 60 mL saline for 1 minute.

**Animal Treatment**

For the bFGF-induced model, after surgery, 60 rats were randomly divided into four groups of 15. The topical normal saline solution, 0.1% DTSH, subconjunctival injections of bevacizumab solution (Genentech, South San Francisco, CA) (2.5 mg/0.1 mL), or DTSH and bevacizumab in combination were administered to the control group, DTSH group, bevacizumab group, and combination group, respectively. The 0.1% DTSH was topically applied four times daily at equal intervals for 6 days. Subconjunctival injections of bevacizumab solution were administered after surgery on days 0 and 5.

In the alkali burn model, after they were burned, 132 rats were randomly divided into four groups of 33. The topical normal saline solution, 0.1% DTSH, subconjunctival injections of bevacizumab solution (2.5 mg/0.1 mL), and DTSH bevacizumab in combination were administered to the control group, the DTSH group, the bevacizumab group, and the combination group, respectively; 0.1% DTSH was topically applied four times daily at equal intervals for 21 days. Subconjunctival injections of bevacizumab solution were administered after surgery in the upper and lower subconjunctiva and then twice a week for 3 weeks.

**Clinical Evaluation**

Slit lamp examinations were performed in a blinded manner once per day throughout the course of the study. Eyes were examined for the presence of corneal epithelial defects, corneal ulceration, corneal perforation, and CNV and other related adverse complications. Corneal epithelial defects were determined using 1.0% fluorescent dye, and the healing time was recorded. Ulcer formation was defined when the epithelial defect persisted and the stroma became involved. The occurrence of corneal ulceration and the healing time were recorded.

**Quantification of Corneal Neovascularization**

In the bFGF-induced CNV model, CNV was quantified as in our previous study. In brief, 6 days after cornea pocket surgery, the rats were euthanized. Ten milliliters of Higgins waterproof India ink (Sanford, Bellwood, IL) was injected to visualize corneal vessels. The eyes were enucleated and fixed in 10% neutralized buffered formaldehyde, and the corneas were dissected and mounted on slides. Slide-mounted corneal images were taken using a slit lamp microscope (SL120; Carl Zeiss, Oberkochen, Germany) with a 25× objective magnification. The CNV length and area were measured (Image Pro-Plus 5.1; Media Cybernetics Company, Silver Spring, MD).

In the alkali burn model, quantification of CNV was also described in detail in our previous study. In brief, the length and area of CNV were measured from the edge of the sclera with a reticle on days 3, 7, 14, and 21 after burn. The area (A) of corneal NV was calculated using the following formula: $A = C/12 \times 3.1416 \times (r - r^2)^2$, where C is the clock hours of corneal NV coverage on the cornea, I is the average length of the selected vessels, and r is the radius of the rat cornea ($r = 3$ mm).

**Real-Time PCR**

Three randomly selected rats from each group were euthanized on day 3 and day 7 in the bFGF-induced CNV model and in the alkali burn model, respectively. Corneas were obtained for further analysis.

Total RNA was extracted with a mini kit (RNeasy; Qiagen, Valencia, CA), and cDNA was generated using a reverse transcription kit (Omniscript RT; Qiagen). MMP2, MMP9, VEGF, and IL-1β mRNA expression was quantified with SYBR Green (Absolute SYBR Green ROX mix; Thermo Scientific, Waltham, MA). The samples were run in triplicate, and the relative expression of MMP2, MMP9, VEGF, and IL-1β was determined by normalizing the expression of each target to β-actin using the $2^{-ΔΔCt}$ method. Primer sequences were as follows: β-actin, 5'-GAAATCGTGGTGAGAACATAGG-3' and 5'-GGCGGAGTGGGCATCTG-3'; MMP2, 5'-GCCACTAAGGGAGATGACATG-3' and 5'-TCTTCTGCACCCAACATGATG-3'; MMP9, 5'-CGTGGTCTCCTCTCATTCACTCT-3' and 5'-CCCTGGAGACTCTTATTTCCAA-3'; VEGF, 5'-TGTCGGGCGTCTGCAATGAT-3' and 5'-TGTCGGTGCTTGGTGAAGTTGA-3'; IL-1β, 5'-GGAGAGTTGATCCAAACAA-3' and 5'-AAACCTCAGTTCGTTGTGACT-3'.

**Matrix Metalloproteinase Activity Assays**

MMP activity was measured by a fluorogenic peptide substrate (R&D Systems) used to assess MMP activity (MMP2 and MMP9) by the protocol recommended by the manufacturer. Briefly, the MMP substrate was diluted in TCN buffer (50 mMol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L CaCl2; pH 7.5) and was added to the supernatants (preincubated with aminophenylmercuric acetate for 1 hour) before incubation at 37°C. After 30 minutes, total MMP activity was determined on a fluorometer (FLX 800 Microplate Fluorescence Reader; Bio-Tek Instruments, Winooski, VT).

**Western Blot Analysis and ELISA**

Rat cornea homogenates (50–100 μg of total protein) were separated in polyacrylamide-SDS gel and electrophoretically onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with TBS/5% nonfat dry milk, the membrane was incubated with antibody against rat iNOS.
and tyrosine-phosphorylated VEGFR1 and VEGFR2, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody, and the signals were visualized by enhanced chemiluminescence detection (Pierce, Rockford, IL). The blots were also reprobed with a specific antibody against β-actin (Sigma). IL-1β concentration in corneal lysates of rat was detected using ELISA kits (eBioscience, San Diego, CA).

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS 16.0, Inc.; Chicago, IL). Statistically significant differences were defined as P < 0.05. Experimental data were compared using one-way ANOVA, independent two-sample t-tests, and χ²/Fisher exact test.

RESULTS

DTSH Enhances the Inhibitory Effects of Bevacizumab on CNV Induced by bFGF

In the bFGF-induced CNV model, no corneal epithelial defect, corneal ulcer, conjunctival necrosis, or other adverse complications related to topical DTSH or bevacizumab were observed in any animal. After 6 days, all tested animals were euthanized to quantify angiogenesis. Typical images of the rat corneas are shown in Figure 1A. Our results show that topical bevacizumab, DTSH or their combination had significantly inhibitory effects on CNV (P < 0.01 by t-test) (Figs. 1B, 1C). When compared with topical bevacizumab or DTSH alone, the combination therapy was more effective at inhibiting CNV (P < 0.01 by t-test) (Figs. 1B, 1C). Using 1.0% fluorescence dye, no angiogenesis was detected in any tested cornea.

DTSH Enhances the Inhibitory Effects of Bevacizumab on CNV Induced by bFGF by Modulating MMP Activity and the Expression of MMPs, VEGF, and Phosphorylated VEGFR

To explore the mechanisms of combination therapy in bFGF-induced CNV model, first we asked whether VEGF or other proangiogenic factors were involved in the bFGF-induced CNV model. Our results showed that doxycycline inhibited VEGF expression in local cornea (Fig. 3A). This result, combined with the aforementioned doxycycline-mediated inhibition of MMP activity and expression and previous studies that showed MMPs enhance VEGF release and modulate VEGF expression,29–33 indicated that doxycycline inhibited the expression of phosphorylated VEGFR1 and VEGFR2 possibly by modulating the MMP-VEGFR pathway. Taken together, this evidence supports our hypothesis.

VEGF and MMPs are involved in the bFGF-induced CNV model. (A) VEGF expression and (B) MMP2 and MMP9 expression were analyzed by real-time PCR in normal cornea or corneas in which bFGF pellets were implanted on day 3 after surgery. Results are reported as mean ± SEM. **P < 0.01.
Effects of Combination Therapy on CNV and CWH in the Alkali Burn Model

To further confirm the effects of combination therapy on CNV and to evaluate its effects on CWH, we used a serious alkali burn model of rats. All corneas developed epithelial defects immediately after alkali burning. The results of epithelial fluorescence dye show significant overall differences in epithelial healing time among the four treatment groups (Fig. 4A) (P < 0.001, ANOVA). Mean epithelial healing times in the untreated control, bevacizumab, doxycycline, and combination groups were 5.20 days, 8.00 days, 3.47 days, and 5.03 days, respectively (Fig. 4A). The topical bevacizumab group had significantly delayed epithelial healing time compared with the untreated group (P < 0.031, t-test). Compared with topical bevacizumab, the combination therapy had significantly decreased epithelial healing time (P < 0.018, t-test).

Figure 3. Combination therapy inhibits bFGF-induced CNC by regulating the expression or activity of MMPs and blocking VEGF-VEGFR binding. (A) Expression of MMP2 and MMP9 in the corneas of rats treated with normal saline solution (Control), 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL) (BEVA), or DTSH and bevacizumab in combination (COMB) or in the normal cornea on day 3 after surgery. (B) The activity of MMP2 and MMP9 in each group on day 3 after surgery. (C) The expression of tyrosine-phosphorylated VEGFR1 and VEGFR2 in each group on day 3 after surgery. (D) The expression of VEGF in the corneas of rats treated with normal saline solution (Control), 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL) (BEVA), or DTSH and bevacizumab in combination (COMB) or in the normal cornea on day 3 after surgery. Results are reported as mean ± SEM. *P < 0.05; **P < 0.01.

Figure 4. DTSH promoted delayed wound healing caused by topical bevacizumab. (A) Healing time of corneal epithelium defects in the corneas of rats treated with normal saline solution (control), 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL) (BEVA), or DTSH and BEVA in combination (COMB) in the alkali burn model. (B) Representative images of epithelial defects and corneal ulceration after DTSH, bevacizumab, or their combination. (a) Representative image from a bevacizumab-treated rat on day 7 reveals corneal epithelial defect. (b) Representative image from a combination-treated rat on day 3 reveals a healing corneal epithelial. (c) Representative image from a bevacizumab-treated rat reveals corneal ulceration on day 14. (d) Representative image from a bevacizumab-treated rat reveals corneal perforation on day 14. Results are reported as mean ± SEM. *P < 0.05; **P < 0.01.
When the epithelial defect persisted and the stroma became involved, corneal ulcers were observed under a slit lamp. Topical application of bevacizumab significantly increased the corneal ulceration recurrence rate compared with the untreated group \((P = 0.035, \chi^2 \text{ test})\) (Table 1), whereas combination treatment significantly decreased the corneal ulceration recurrence rate compared with bevacizumab \((P = 0.009, \chi^2)\) (Table 1). Representative images of the rat corneas are shown in Figure 4B.

The length and area of new corneal vessels were observed and measured on days 3, 7, 14, and 21 after alkali burning. The first clinical finding of CNV ingrowth toward the central cornea occurred at day 2, reached its peak by day 17 to 19, and then regressed after 19 days. Significant differences in vessel length (all \(P < 0.01, \text{ ANOVA; days 3–21}\)) and vessels area (all \(P < 0.01, \text{ ANOVA; days 3–21}\)) were found among all groups (Table 2). Compared with control, the bevacizumab, DTSH, and combination treatments significantly reduced CNV in terms of vessel length (all \(P < 0.01, \text{ ANOVA; days 7–21}\)) and vessel area (all \(P < 0.01, \text{ ANOVA; days 7–21}\)). Combination treatment exhibited better effects than either bevacizumab or DTSH alone in reducing vessel length (all \(P < 0.01, \text{ ANOVA; days 7–21}\)) and vessel area (all \(P < 0.01, \text{ ANOVA; days 7–21}\)) (Table 2). Representative images of the rat corneas are shown in Figure 5.

**Effects of Combination Therapy on the Expression and Enzymatic Activity of MMP-2 and MMP-9 in Local Burned Cornea**

Previous studies have demonstrated that doxycycline could effectively inhibit ocular neovascularization and enhance corneal wound healing, by potently inhibiting the activity of MMPs. Thus, next, we asked whether combination therapy has some inhibitory effects on the expression and enzymatic activity of MMP-2 and MMP-9 in local burned cornea. Real-time PCR results showed that topical DTSH and bevacizumab reduced MMP-2 and MMP-9 activity compared with the untreated group. By analyzing the enzymatic activity of MMPs, our results showed that topical DTSH significantly inhibited MMP-2 and MMP-9 activity compared with the untreated group (Fig. 6B).

**Doxycycline Decreases the Expression of iNOS and the Production of IL-1\(\beta\) in Local Burned Cornea**

Because nitric oxide and IL-1 also play important roles in both CNV and CWH, we next explored the role of doxycycline on iNOS expression and IL-1\(\beta\) production in the alkali-burned cornea. Western blot analysis and real-time PCR results showed that topical doxycycline significantly reduced the expression of iNOS and IL-1\(\beta\) (Figs. 7A, 7B) compared with the untreated group. ELISA results demonstrated that DTSH treatment potently inhibited the production of IL-1\(\beta\) in burned corneas (Fig. 7C).

**DISCUSSION**

During CNV, VEGF is regarded as the most important proangiogenic factor because it increases vascular permeability, proliferation, migration, differentiation, and tube formation and inhibits the apoptosis of vascular endothelial cells. Inhibiting VEGF activity with bevacizumab is a promising approach in the treatment of CNV, and off-label bevacizumab has been used to treat clinical patients. However, recent studies have found that topical bevacizumab leads to corneal thinning and delays CWH. In addition, topical bevacizumab alone is insufficient for the profound inhibition of CNV. Therefore, it is necessary to explore a method to reduce the complications and to promote the therapeutic effects of bevacizumab for clinical applications. Doxycycline has been successful as a nonselective, broad-spectrum inhibitor of MMP in the treatment of a variety of disorders. Unlike corticosteroids and bevacizumab, which inhibit CNV but delay CWH, the unique superiority of doxycycline is that it inhibits CNV and enhances CWH. Therefore, we proposed that the combination of topical DTSH and bevacizumab would enhance the therapeutic effects of bevacizumab and prevent its side effects on CWH. Using bFGF-induced CNV and alkali burn models, we found that the combination treatment enhanced both the inhibitory effects of bevacizumab on CNV and the promotion of delayed CWH caused by bevacizumab, which supports our hypothesis.
MMPs are among the most potent proangiogenic regulators, and they play important roles in CNV.\(^1\) MMPs can facilitate the angiogenic factor–stimulated migration of endothelial cells by disrupting cell–cell and cell–extracellular matrix connections, ultimately leading to the formation of new vasculature.\(^2\) More important, the synergistic actions of VEGF and MMPs have been found in angiogenesis.\(^29\) VEGF increases MMP release and decreases tissue inhibitors of metalloproteinases release, whereas MMPs activate the angiogenic activity of VEGF and induce VEGF release.\(^29\) MMPs also play important roles in CWH.\(^16\) Many studies have confirmed that inhibiting the activity of MMPs promotes CWH.\(^16\) In the present study, our results showed that topical DTSH inhibited the expression and enzymatic activity of MMP-2 and MMP-9 in both bFGF-induced CNV and alkali burn models. Our results are consistent with previous studies and suggested that inhibiting the expression and activity of MMPs by doxycycline may play important roles in enhancing the therapeutic effects of bevacizumab on CNV and the promotion of delayed CWH by bevacizumab. Of note, in the present study, we showed that topical bevacizumab suppressed bFGF-induced CNV and that DTSH combined with topical bevacizumab improved the bevacizumab-mediated therapeutic effects on bFGF-induced CNV. Consistent with our study, Chen et al.\(^5\) showed that bevacizumab was able to inhibit VEGF-induced CNV and bFGF-induced CNV in a pellet model. However, the mechanisms of bevacizumab-mediated inhibitory effects in bFGF-induced CNV remain to be investigated. Our study demonstrated that VEGF and MMP are involved in bFGF-induced CNV. Next, we found that topical DTSH and bevacizumab could inhibit MMP activity and MMP expression induced by bFGF. Interestingly, we found that DTSH treatment suppressed the phosphorylation of VEGFR1 and VEGFR2 and enhanced bevacizumab-mediated inhibition of VEGFR1 and VEGFR2 (Fig. 3C). We next explored mechanisms in DTSH treatment to suppress the phosphorylation of VEGFR1 and VEGFR2 by testing whether DTSH treatment can inhibit the expression of VEGF, which can induce the tyrosine phosphorylation of VEGFR. Our results showed that DTSH treatment reduced VEGF expression. This finding indicated that DTSH treatment suppressed the phosphorylation of VEGFR1 and VEGFR2 by possibly inhibiting the expression of VEGF. Consistent with our study, previous studies reported that doxycycline could reduce VEGF production in hydrocele patients and lymphatic filariasis patients.\(^43\) MMP can enhance VEGF release and modulate VEGF expression.\(^29\) This evidence, combined with the aforementioned doxycycline-mediated inhibition of MMP activity and expression, indicated that doxycycline suppresses the phosphorylation of VEGFR1 and VEGFR2 possibly by modulating the MMP-VEGF-VEGFR path.

**Figure 5.** Typical images of corneal neovascularization in the corneas of rats treated with normal saline solution, 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL), or DTSH and bevacizumab in combination on day 7 after corneal alkali burn. Red arrows: iris; white arrows: new corneal vessels.

**Figure 6.** Doxycycline inhibits the expression and activity of MMP2 and MMP9 in local burned cornea. (A) The expression of MMP2 and MMP9 in the corneas of rats treated with normal saline solution (Control), 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL) (BEVA), or DTSH and bevacizumab in combination (COMB) or in normal cornea on day 7 after corneal alkali burn. (B) The activity of MMP2 and MMP9 in each group on day 7 after corneal alkali burn.
way and may explain, at least in part, the mechanism of synergistic effects of combination treatment.

When the cornea is injured, corneal epithelial cells and other local cells immediately release a larger number of mediators, including NO, IL-1, and MMPs, to recruit inflammatory cells into the local injured cornea. Therefore, 12 to 24 hours after the injury, inflammatory cells arrive in the local cornea and release inflammatory mediators to contribute to the development of corneal disorders. Thus, the inhibition of inflammation is also very important in the treatment of CNV and CWH. For example, previous studies have shown that reducing iNOS expression and IL-1 production or blocking the IL-1 receptor inhibits CNV and promotes CWH. Previous studies have also shown that doxycycline inhibits iNOS expression and IL-1 production. Thus, in this study, we further explored the effect of doxycycline on iNOS expression and IL-1 production in burned corneas. Topical doxycycline potently inhibited iNOS expression and IL-1β production. Therefore, inhibiting iNOS expression and IL-1β production could also play important roles in the doxycycline-mediated effects on CNV and CWH.

In summary, our study demonstrated for the first time that the combination of topical application of doxycycline and bevacizumab had stronger therapeutic effects than either bevacizumab or DTSH alone on CNV induced by bFGF or alkali burn in rats. Furthermore, in the alkali burn model, our results showed that topical DTSH not only enhanced the inhibitory effects of bevacizumab on CNV, it also promoted delayed CWH caused by bevacizumab. Therefore, the combination of topical doxycycline and subconjunctival bevacizumab could be a good alternative treatment for the clinical management of CNV.

References


**Figure 7.** Doxycycline decreases the expression of iNOS and the production of IL-1β in local burned cornea. (A) iNOS expression was analyzed by Western blot in the cornea of rats treated with normal saline solution (Control), 0.1% DTSH, bevacizumab (2.5 mg/0.1 mL) (BEVA), or DTSH and bevacizumab in combination (COMB) or in normal cornea on day 7 after corneal alkali burn. IL-1β production was analyzed by real-time PCR (B) and ELISA (C) in each group on day 7 after corneal alkali burn. Results represent three independent experiments (mean ± SEM). *P < 0.05; **P < 0.01.
Synergistic Effects of Doxycycline and Bevacizumab on CNV


