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Original Article

Thymoquinone (TQ) inhibits corneal neovascularization in a rabbit model

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Abstract: To assess the inhibitory effect of thymoquinone (TQ) on corneal neovascularization (CNV) in rabbits and determine the effective concentration. A total of 30 rabbits subjected to alkali burn injury were randomly divided into 3 groups, and treated with 0.8% TWEEN 20 (Group T), 0.1% (Group 0.1), and 0.4% (Group 0.4) TQ eye drops, respectively. Another group of 5 animals received no treatment and were used as controls (Group C). The inhibitory effects of drugs on CNV were evaluated by the percentage of cornea area covered by neovascularization and VEGF expression levels (immunohistochemistry and RT-PCR). The corneal structure in group C was normal. In the TQ treatment groups (0.1 and 0.4%), the values of CNV percent area at 7, 14, 21, and 28 days varied in a time-dependent manner. Interestingly, at all time points, means CNV percent area obtained for Groups 0.1 and 0.4, respectively, were reduced compared with those of Group T. In agreement, immunohistochemistry and RT-PCR results showed undetectable corneal VEGF expression in the control group; in addition, corneal VEGF expression levels were significantly higher in Group T compared with TQ treatment groups, with lowest values obtained in Group 0.4. TQ inhibits CNV in rabbits after alkali burn injury, and most effectively at 0.4%.

Keywords: Corneal neovascularization, VEGF, thymoquinone, rabbit, animal model

Introduction

Corneal chemical burns and severe keratitis can all cause corneal neovascularization (CNV), which in turn results in the loss of normal corneal transparency and decreased vision, and even blindness [1]. CNV involves many pathological agents such as VEGF, a well-known angiogenic factor, whose family members include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and the placenta growth factor (PIGF). VEGF-A plays a key role in vascular development, and its expression is positively correlated with the number of new corneal blood vessels [2]. High VEGF-A levels activate the VEGF-dependent protein kinase B (AKT/PKB) and extra cellular signal-regulated kinase (ERK) signaling pathways, which promote the differentiation, proliferation, and migration of vascular endothelial cells, and increase vascular permeability [3, 4]. In addition, VEGF interacts with many mediators, including MMP, interleukin, COX-2, and iNOS, which are positively correlated with its expression [5]. VEGF is also regulated by NF-κB at the transcription level [6]. Interestingly, VEGF mRNA and protein expression, as well as VEGF receptor levels, were shown to be significantly increased in infiltrated inflammatory cells and endothelial cells of new blood vessels; when VEGF stimulates extracellular matrix secretion by corneal stromal cells, vascular endothelial cell activity also increased [7]. Considering its complex mechanism and clinical significance, it is urgent to identify safe and effective treatments for CNV.

Thymoquinone (TQ) is a bioactive substance extracted from the seed oil of *Nigella sativa*, which has been shown to satisfactorily inhibit various proliferative tumors [8-10], and was proposed to inhibit angiogenesis or neovascularization [11]. However, the inhibitory effect of TQ on CNV has been reported only once [12]. TQ ($C_{10}H_{12}O_2$; molecular weight of 164.2) is obtained from *Nigella sativa*'s seed oil. Because TQ has anti-inflammatory and immune-boosting effects [13], it has attracted increasing attention, with most studies assessing its anti-tumor effects [14].

In addition, TQ was shown to have a very strong anti-inflammatory activity, through NF-κB pathway inhibition [15-17], nuclear translocation of the p56 protein, and reduced COX-2 gene expression [15] as well as MMP-2 and -9 activities [18]. The anti-proliferation mechanism of TQ is associated with mitogen-activated protein kinase (MAPK) and protein kinase B (AKT/PKB), reducing the proliferation of multiple myeloma, squamous cell carcinoma, and human prostate cancer cell lines [19, 20]. Yi et al. [11] showed that neovascularization is reduced by TQ via induction of AKT and ERK, two signaling pathways needed for vascular endothelial cell activation. However, these authors found that TQ only slightly inhibits VEGFR-2, suggesting that TQ does not directly affect this molecule; instead, TQ was suggested to affect the VEGF-dependent ERK pathway, thus reducing VEGF expression.

The present study aimed to assess the inhibitory effect of TQ on CNV in rabbits and determine the optimal effective concentration.

Materials and methods

Animals

A total of 35 healthy New Zealand white rabbits of both sexes, provided by the Experimental Animal Center of Shanxi Medical University, were used in this study; they were 6 to 9 months old and weighed 2.0-2.5 kg. Rabbit corneas were examined using a slit lamp microscope, and no abnormality was found. All experimental procedures were in accordance with the Experimental Animal Regulations of the National Science and Technology Commission and approved by the local ethics committee.

Drugs and reagents

TQ powder was purchased from Tokyo Chemical Industry Co., Ltd. (Toshima, Kita-Ku, Tokyo, Japan) and resuspended in 0.8% Tween 20 at 3 different doses. Anti-rabbit VEGF polyclonal primary antibody was from Beijing Biosynthesis Biotechnology Co., Ltd. (China, bs-1313R). The secondary antibody reagent kit (rabbit immunohistochemistry reagent kit, sp-0023) was purchased from ZYMED (USA). The DAB chromogenic agent was obtained from ZSGB-BIO (Beijing, China).

Establishment of the animal model and treatment

Three days before operation, 30 rabbits received 0.5% levofloxacin drops in the eyes, 4 times daily, to prevent infection. Before surgery, general anesthesia was performed by ear vein injection of 30 g/L pentobarbital sodium (30 mg/kg), and topical anesthesia was carried out with 0.4% oxybuprocaine hydrochloride eye drops. A round, 5.0 mm single layer filter was saturated with 1 mol/L sodium hydroxide solution, and placed in the center of the rabbit cornea for 15 s; then, cornea and conjunctiva sac were washed with normal saline for 1 min to form a disc-shaped burn white area with a clear edge [21]. The burn was confirmed as moderate alkali burn, according to the Hughes indexing method [22]. Three different doses of TQ eye drops were prepared using 0.8% Tween 20. The 40 model rabbits were randomly divided into 3 groups (n=10), and treated with 0.8% TWEEN 20 (Group T), and 0.1 (Group 0.1) and 0.4% (Group 0.4) TQ eye drops, respectively. Another group of 5 animals received no treatment and were used as controls (Group C). Rabbits were treated with the corresponding drug 4 times daily. Each animal also received 0.5% levofloxacin eye drops 4 times/day after surgery for 1 week to prevent infection.

Sample preparation

All rabbits were sacrificed using the air embolism method 28 days after alkali burn injury. Corneal tissues with sclera (≥1 mm wide) were collected under sterile conditions. Half of the samples were fixed in 10% neutral formalin, embedded in paraffin, and serially sectioned perpendicularly to the cornea surface to a thickness of approximately 5 μm. The sections were analyzed by immunohistochemistry. The other half of the corneal tissues was used for RT-PCR assays.

Observation parameters

Calculation of CNV length and area: The CNV length was measured under a slit lamp after 1, 7, 14, 21, and 28 d. The CNV growth area was derived using the following formula: $S = C/12 \times 3.11416 \times [r^2 - (r - l)^2]$. The term S represents CNV growth area; C is the number of clock hours around the circumference of the CNV

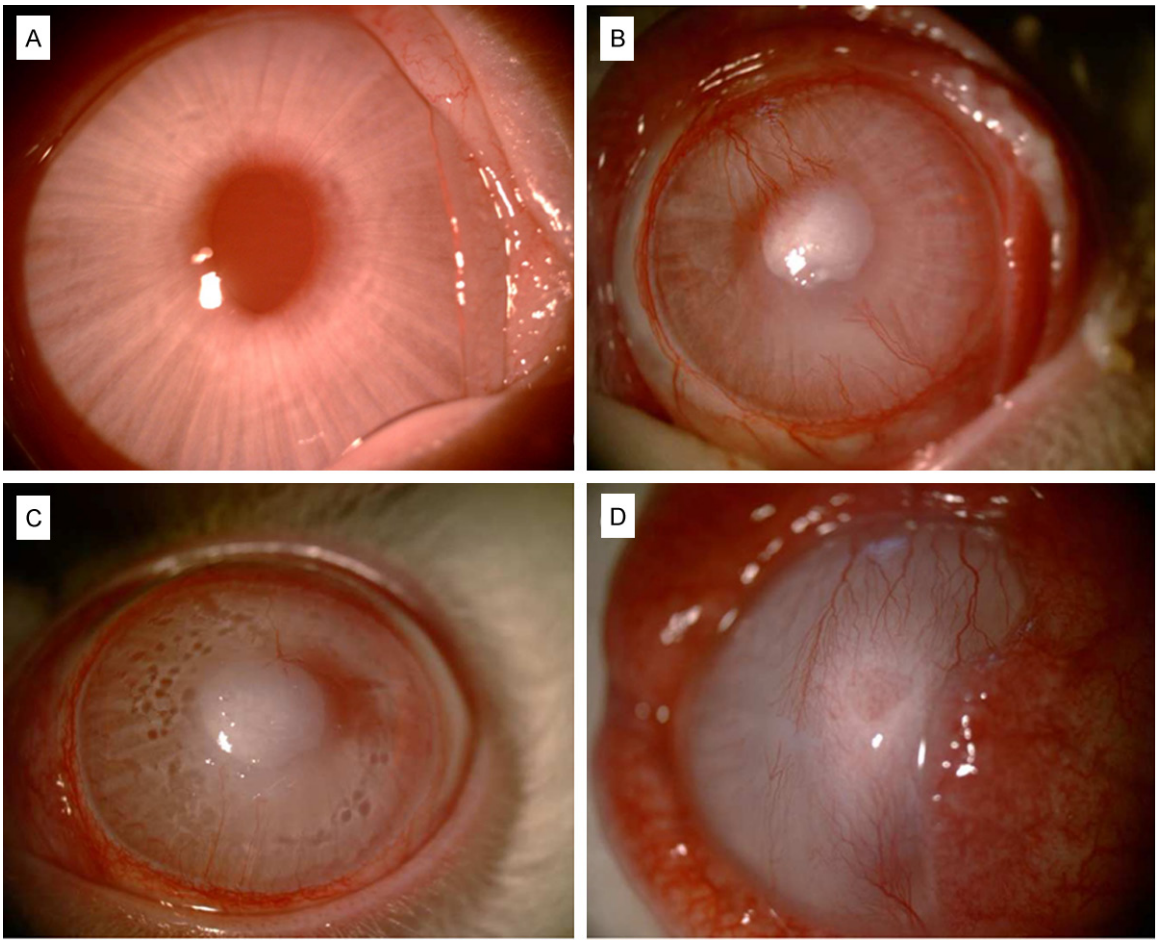

Figure 1. Morphological characteristics of CNV at 28 days of medication. A. In Group C, corneas were transparent with no neovascularization. B. In Group 0.1, the new blood vessels retreated, their diameters were decreased, and they showed a sparse distribution. C. In Group 0.4, most capillaries had already retreated, with only a few residual blood vessels remaining. D. In Group T, the new blood vessels became thicker and were branched, with significant bifurcations on the top ends; a depressed area occurred in the center of the cornea, and the grey-white corneal opacity was further aggravated.

network; r is the corneal radius; l represents the CNV length.

Immunohistochemical detection of VEGF expression: VEGF expression was assessed by the streptavidin-peroxidase conjugated method (S-P method). Slides were baked at 60°C for 2 h, deparaffinized, and rehydrated. Antigen retrieval was performed in boiling citrate buffer for 15 min; after cooling down to room temperature, slides were blocked in 3% H_2O_2 for 20 min and placed in normal goat serum (37°C, 20 min). Afterwards, slides were sequentially incubated with primary (overnight at 4°C), secondary (37°C, 20 min) and tertiary (horseradish peroxidase labeled streptavidin, 37°C, 20 min) antibodies. Development was carried out using 3,3'-diaminobenzidine (DAB) and slides were counterstained with hematoxylin. Finally, slides were mounted using neutral balsam, and observed under a light microscope. VEGF-positive expression was calculated by computer image analysis (using Olympus CellSens Dimension image analysis software at 400 ×). The primary antibody was replaced by the antibody dilution solution in negative controls; a colon cancer sample was used as positive control. Samples were analyzed by technicians blinded to the experiment.

Assessment of VEGF mRNA expression by RT-PCR: Total RNA was prepared from rabbit cornea samples using TRIzol Reagent (Life Technologies, USA) according to the manufac-

Table 1. Comparison of CNV in experimental and control groups (mean ± SD, %)

	Group 0.1% TQ	Group 0.4% TQ	Group T
7 d	40.43±3.37[a]	28.78±4.63[a]	78.74±2.64
14 d	51.81±2.75[a,b]	31.87±5.42[a,b]	135.93±5.29
21 d	44.39±5.86[a,b,c]	26.37±4.82[a,b,c]	153.52±5.25
28 d	30.76±5.93[a,b,d]	19.17±1.90[a,b,d]	140.87±2.65

F^{group}=214.572, P<0.01; F^{time}=5.228, P<0.01. [a]P<0.05 versus the respective group T, [b]P<0.05 versus the respective day 7 value, [c]P<0.05 versus the respective day 14 value, and [d]P<0.05 versus the respective day 21 value (ANOVA, LSD test).

turer's instructions. Reverse transcription was carried at as follows: 11 μl of RNA (1 μg) was mixed with 1 μl random primers (0.2 μg/ml) and incubated at 65°C for 5 min; then, 4 μl of buffer solution, 3 μl of dNTP mix (10 mmol/L), l μl of RNase inhibitor (20 U/μl), and l μl of reverse transcriptase (20 U/μl) were added and incubated sequentially at 25°C for 10 min, 42°C for 1 h, and 72°C for 15 min.

For RT-PCR, the reaction mixture (20 μl) comprised the following: 10 μl of FastStart Universal SYBR Green Master (ROX), 0.5 μl of upstream primer 5CAGCAGTCGTTGGAGCGAGCAT3 (15 μM), 0.5 μl of downstream primer 5TCACATGGCATCTCACGATATTTGG3 (15 μM), 2 μl of cDNA, and 7 μl of DNase/RNase-free water. RT-PCR was carried out as follows: initial denaturation at 94°C for 10 min; 45 cycles of 94°C for 15 s and 60°C for 60 s. The CT (cycle threshold) values were obtained, and VEGF mRNA expression levels and fold change determined relatively to control samples.

Statistical methods

The data are presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) and LSD as post-hoc were used to compare CNV areas, positive VEGF expression rates on immunohistochemistry slides, and relative VEGF mRNA expression levels. Statistical analyses were performed using the SPSS 18.0 software. P<0.05 was considered statistically significant.

Results

Macroscopic characteristics of CNV

In Group C, corneas were transparent with no neovascularization. The corneas in the other groups that received alkali burn injuries were affected. At 1 day, edema was observed in both the cornea and conjunctiva, the latter showing overt hyperemia. At 3 days, CNV was observed in Groups 0.1, 0.4, and T. In Group 0.1, the new vascular buds were very dense with centripetal growth, and involved less than half a quadrant of the cornea. From 14 to 21 days, the new vessels became thicker and showed a weeping willow shape, with bifurcations on the top ends. At 28 days, blood vessels had retreated, with decreased diameters and sparser distribution. In Group 0.4, the new vascular buds were short and thin at 7 days. At 14 days, blood vessels in some areas had retreated, but some residual new blood vessels were still growing. At 28 days, most blood vessels had already retreated, with only few residual blood vessels remaining. In Group T, blood vessels showed centripetal growth from the corneal limbus to the cornea center at 3 days. At 14 days, blood vessels had not significantly retreated, thus remaining dense and vigorously growing. At 21 days, blood vessels nearly occupied the entire cornea, with a mesh pattern. Finally, at 28 days, the new blood vessels became thicker and showed a branched shape, and significant bifurcations were observed on the top ends; the grey-white corneal opacity was further aggravated (**Figure 1**).

The means percent area of CNV at 7, 14, 28 days in Groups 0.1 and 0.4 varied in a time-dependent manner (F^{group}=214.572, P<0.01; F^{time}=5.228, P<0.01). Interestingly, CNV lengths and areas in Groups 0.1 and 0.4 were smaller compared with the Group T. A comparison of average CNV areas in various groups yielded the following relationship: Group 0.1> Group 0.4 (**Table 1**).

VEGF protein expression in corneal tissues

Positive VEGF expression was detected immunohistochemically as brown or brown-yellow granules in the cytoplasm. The VEGF positive samples were separated into + (weakly positive), ++ (positive), and +++ (strongly positive). No VEGF expression was observed in Group C. The other groups showed different degrees of VEGF expression in corneal epithelial and stromal cells, inflammatory cells, and endothelial cells of new blood vessels in Groups 0.1, 0.4 and T. In Groups 0.1, VEGF expression in the cytoplasm of corneal epithelial cells was obvi-

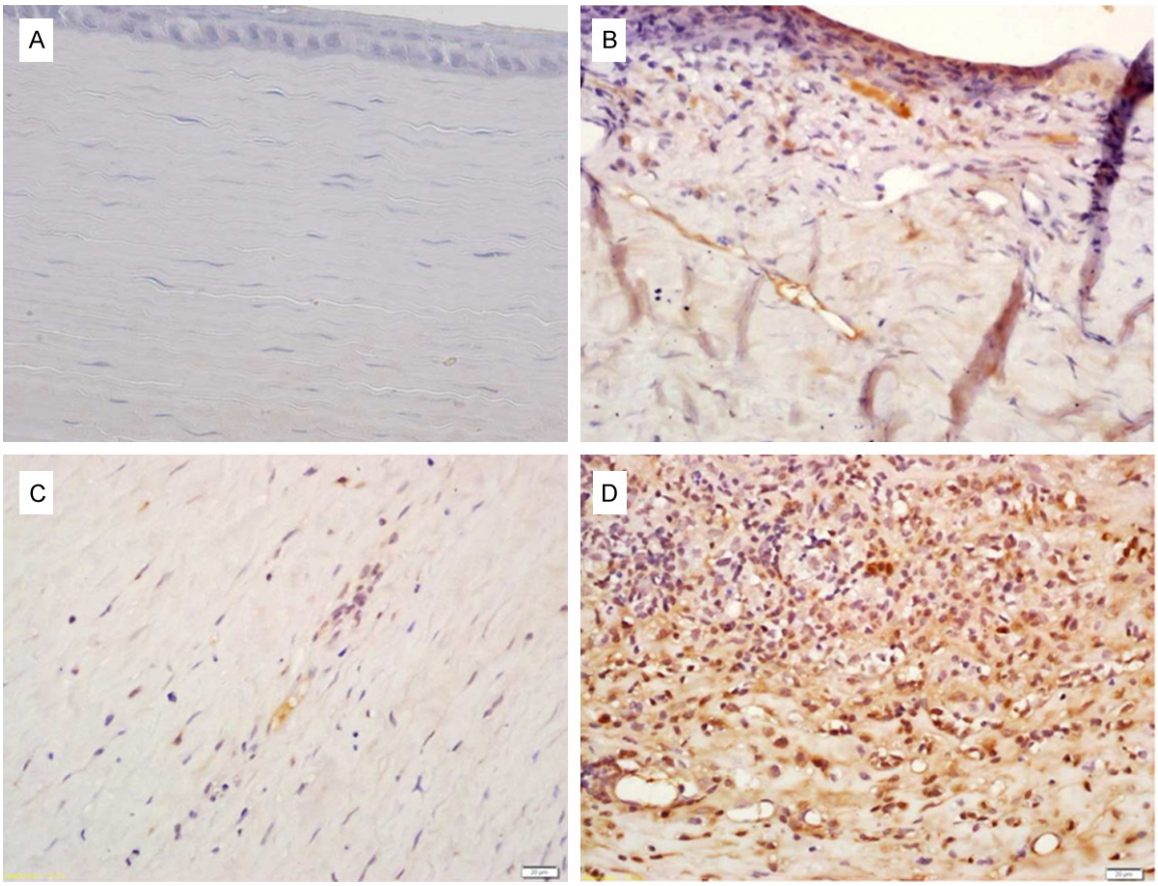

Figure 2. VEGF protein expression in corneal tissues assessed by immunohistochemistry. A. In Group C, no VEGF expression was detected. B. In Group 0.1, yellow granules were observed in the cytoplasm of some lymphocytes; endothelial cells in small blood vessels also showed positive expression. C. In Group 0.4, a weak VEGF-positive expression was observed in the cytoplasm of a few inflammatory cells and vascular endothelial cells in the stromal layer. D. In Group T, the tissue structure of the stromal layer was disordered, with loose arrangement; a prominent staining was found in the cytoplasm of a large number of neutrophils, lymphocytes, plasma cells, and vascular endothelial cells, indicating strongly positive VEGF expression.

ous. Inflammatory cells, particularly neutrophils and lymphocytes, were found infiltrated into the superficial stromal layer, and VEGF positive expression was observed in vascular endothelial cell cytoplasm. In Group 0.4, only basal epithelia cell cytoplasm was stained, and a weak VEGF-positive result was obtained in the cytoplasm of a few inflammatory and vascular endothelial cells in the stromal layer. In Group T, the cytoplasm of the entire epithelial layer showed stark VEGF expression. Additionally, the tissue structure of the stromal layer was disordered and loosely arranged, with large mature blood vessels observed; staining was also prominent in the cytoplasm of a large number of neutrophils, lymphocytes, plasma cells, and vascular endothelial cells (**Figure 2**).

Computer image analysis showed VEGF-positive expression of 0, 0.38±0.12, 0.24±0.13 and 0.56±0.04 in Groups C, 0.1, 0.4 and T, respectively. The differences among the 4 latter groups were statistically significant ($F=16.23$, $P<0.01$). A comparison between Group T and each TQ group yielded a *P* value of less than 0.01, indicating a statistically significant difference. A statistically significant difference was obtained for Groups 0.4 and 0.1 ($P<0.05$) (**Figure 3**).

VEGF gene expression levels

The relative expression levels of VEGF mRNA were determined by RT-PCR, as 0, 1.68±0.45, 1.18±0.18 and 5.01±1.64 in Groups C, 0.1, 0.4 and T, respectively. Statistically significant differences were obtained between the TQ groups and Group T ($F=37.054$, $P<0.01$). Groups 0.1 and 0.4 were significantly different ($P<0.05$) (**Figure 4**).

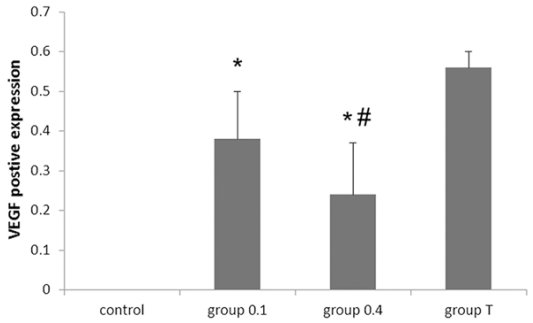

Figure 3. Quantitation of VEGF-positive expression by immunohistochemistry. Groups 0.1 and 0.4 received eye drops containing 0.1 or 0.4% TQ, respectively. Group T received 0.8% Tween 20. *P<0.05 *vs* group T, #P<0.05 *vs* group 0.1.

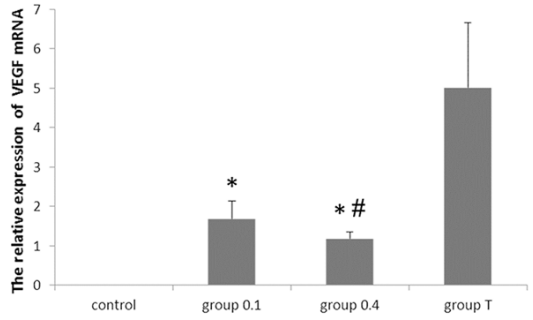

Figure 4. Quantitation of VEGF mRNA levels by RT-PCR. Groups 0.1 and 0.4 received eye drops containing 0.1, or 0.4% TQ, respectively. Group T received 0.8% Tween 20. *P<0.05 *vs* group T, #P<0.05 *vs* group 0.1.

Discussion

CNV is a complex event involving VEGF-A, a key factor for vascular development (Takahashi 2011). The expressions of the VEGF mRNA and protein, as well as the expression of the VEGF receptor, are significantly increased in infiltrated inflammatory cells and in the endothelial cells of new blood vessels; when VEGF stimulates extracellular matrix secretion by corneal stromal cells, the activities of the vascular endothelial cells are also enhanced [7].

TQ has been used as a medicine for more than 2000 years, and is one of the most frequently used medicinal herbs in folk medicine of Mediterranean and West Asian regions. Thanks to its anti-inflammatory and immune-boosting effects [13], TQ has been intensively studied in the past years: more than 345 hits are found in PubMed with the keyword "thymoquinone", among which 70% were published in the past 5 years and more than a third assessing its anti-tumor effect [14].

The present study showed active CNV in the model animals treated with Tween 20 only at 7-14 days. At 28 days, blood vessels were retreating in Group T, and CNV area decreased, which is basically consistent with the morphological changes and pathological staging of clinical CNV [23]. Compared with Group T, animals treated with TQ showed more prominent capillary retreat and decreased vascular density. In these animals, CNV areas were smaller than those obtained in Group T. In agreement, VEGF-positive rates in TQ groups were significantly lower compared with values obtained for Group T, as shown by immunohistochemistry; in addition, VEGF mRNA levels were lower in TQ groups in comparison with Group T. These results indicate that TQ effectively reduces VEGF expression in rabbit tissues after alkali burn injury to the cornea. This effect may occur through inhibition of the VEGF-dependent ERK and NF-κB pathways [3, 4], causing the down-regulation of cellular inflammatory mediators, and thus significantly reducing neovascularization. Here, we assessed three different TQ concentrations for their effects on CNV. TQ's inhibition of CNV was dose dependent. CNV areas in animals treated with 0.4% TQ were the least. Furthermore, the VEGF-positive rates (immunohistochemistry) and relative VEGF mRNA levels (RT-PCR) were both lowest on average in animals treated with 0.4% TQ.

TQ is a drug with great potential. By assessing CNV produced after alkali burn injuries in rabbits and the resulting VEGF expression in corneal tissues, we found that TQ effectively inhibits CNV. These results corroborate previous findings that TQ has an inhibitory effect, comparable with that of triamcinolone, on corneal neovascularization in rats [12]. We also found that 0.4% TQ eye drops most effectively inhibits CNV occurrence and development. However, the mechanism underlying this effect is unclear and requires more detailed studies before the clinical application of TQ.

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Disclosure of conflict of interest

None.

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