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Full Length Research Paper

Effect of tetrandrine on the expression of leptin (LP) and vascular endothelial growth factor (VEGF) in corneal neovascularization of rats

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To investigate the effect of tetrandrine (Tet) on the expression of leptin (LP) and vascular endothelial growth factor (VEGF) in corneal neovascularization (CNV). CNV model was made by alkaline burning. The expressions of LP and VEGF in CNV before and after using Tet were detected slit lamp biomicroscopy, photography and immunohistochemical staining. Immunohistochemistry showed LP was observed in the epithelium, stroma and endothelium of normal rat corneas. The epithelial and stromal layers of the normal rat corneas demonstrated minimal or no VEGF expression. The expression of CNV increased markedly throughout the whole cornea. In the Tet experimental group there were weak (*P<0.05*) expression levels for LP and VEGF in the corneal stroma. Tet can effectively inhibit the growth of CNV after alkali burns in rats, and reduce the expression of LP and VEGF in CNV.

Key words: Alkali burns, corneal neovascularization, leptin, vascular endothelial growth factor, tetrandrine.

INTRODUCTION

The occurrence of CNV poses a serious threat to the vision. Corneal inflammation, infection, trauma, degeneration and other factors often induce neovascularization in the normal corneal tissue. CNV can damage the normal micro-environment of the cornea so that immune privilege in the anterior segment deviates and then disappears which is a high-risk factor in corneal graft rejection. Moreover, neovascularization structure is more fragile and is easy to penetrate. Bleeding, seepage and secondary fibrosis can lead to CNV, which is the most common cause for blindness. However, there is no specific treatment for CNV. The present work is to investigate the effect of Tet on the expression of LP and VEGF in CNV, which provides laboratory support for the selection of assistant CNV-suppressants in clinical practice.

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Abbreviations: LP, leptin; **VEGF,** vascular endothelial growth factor; **CNV,** corneal neovascularization; **Tet,** tetrandrine; **SD,** sprague dawley; **SABC,** streptavidin-biotin complex; **PBS,** portable batch system; **PLA 2,** phospholipase A2.

MATERIALS AND METHODS

Materials

This experiment utilizes 30 healthy 12 week old SD (Sprague Dawley) rats, each weighing 200 to 250 g. This group of 30 rats was made up of both males and females. These rats are provided by the Department of Laboratory Animal Science of the First Affiliated Hospital of Zheng Zhou University. The rats were randomly divided into three groups: a negative control group A consisting of 5 SD rats, whose corneas are left intact; the remaining 25 SD rats' right eye as the experimental CNV intervention group C, their left eyes as the experimental control group B. The models of severe corneal alkali burns were induced in those 25 rats (Sun et al., 2007) Induce general anesthesia by injecting 10% chloral hydrate (3 ml/kg) intraperitoneally. Use 0.25% tetracaine for corneal surface anesthesia three times. Irrigate the conjunctival sac with a 1:2000 dilution Gentamicin Sodium Chloride twice and use cotton buds to remove the excessive liquid. Immerse single layer round filter papers of unified specification of 3.0 mm diameter into 1mol / L sodium hydroxide solution 1 min, remove and put the filter paper on sterile cotton for 2 sec to remove excessive lye, then place the filter paper in the center of the left eye on the cornea surface for 20 s.

Remove the filter paper and irrigate the conjunctival sac by

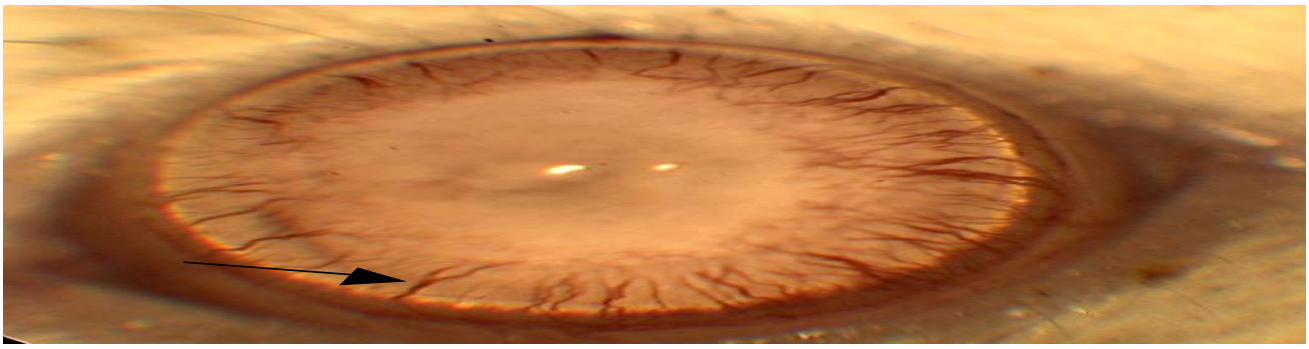

Figure 1. Group B CNV strong on 7 day, has reached the area of corneal burns.

physiological saline solution for 1 min and observe the cornea in the burn area for changes to a dense white round opacity. All model eyes were given 1% atropine. Then the rats are put into cage and fed normally. Rats in control group B are given eye drops of physiological saline solution four times a day from the first day that they got alkali burn. Rats in experimental intervention group C were given Tet (40 mg/kg, Jinhua, Zhejiang pharmaceutical production, powder, purity> 98%) 4 times a day from the first day that they got alkali burn. The above two groups were given 1% atropine once a day 5 min after they got eye drops.

Methods

Observed and measured

Observe and record the change of the rat corneal alkali burn model every day. On 1, 4, 7, 14 and day 21, induce anesthesia by intraperitoneal injection of 10% chloral hydrate (3 ml/kg). Observe the corneal opacity, corneal neovascularization length and conjunctival congestion under a microscope and photograph for group B and group C. The area of CNV is measured by the Robert computer model instruction formula: S = C/12 × 3.14 × [r2-(rl) 2], of which C for the points of the circumference involved by new corneal blood vessels, r for the radius of the cornea, l for the in-depth length of the new blood vessels which grows from the limbal cornea (Seo et al., 2001).

Sample preparation

After measured, the rats in normal control group A, C and B would be killed by deep anesthesia by intraperitoneal injection of over 10% chloral hydrate after 1, 4, 7, 14 and day 21. In relatively sterile conditions, remove the eyes rapidly and, respectively, cut the cornea along the outer edge of 1mm, separate to two parts averagely, and number the different groups. Fix them in 4% paraformaldehyde for 24 h and then embed them in paraffin and cut them into 5 μm thick slices. One half of the specimens were detected by HE staining and observed under light microscopy (magnification according to the reunification of the 10 × 40). The second half of the specimens was left to be detected by immunohistochemical staining.

Immunohistochemistry

Post Corneal alkali burn, embed the second half of the cornea in paraffin and cut into 5 μm thick paraffin sections after 1, 4, 7, 14 and day 21. With the Streptavidin -Using Streptavidin - Biotin - complex (SABC) method, observe and take pictures under light microscopy. According to the company instructions, a 1:100 for the rabbit anti-rat polyclonal LP antibody (purchased from Wuhan Bo Shide Biological Engineering Company Limited), a 1:100 rabbit anti-rat multi-VEGF antibody (purchased from Sequoia in Beijing's Golden Bridge Biological) and a biotinylated goat anti-rabbit IgG are used. An immunohistochemical negative control experiment set up portable batch system (PBS), methods and steps are same as before.

Statistical analysis

The experimental data are analyzed by X^2 test experimental data, Spearman rank correlation test, using STATA 11.0 software for statistical analysis, $P < 0.05$ is statistically significant.

RESULTS

The growth of CNV

A group without the growth of new blood vessels; In B group at the 7th day CNV grows strongly, and has reached the area of corneal burns, the length of blood vessel is (1.75 + 0.06) mm; However in Group C at the 7th day CNV is inhibited significantly and has become sparse. The length of blood vessels is (0.88 + 0.04) mm. The area of CNV group B is (9.88 + 0.22) mm^2, C group is (4.66 + 0.35) mm^2 (Figures 1 and 2). The length of group C of CNV is significantly shorter than that of group B .The data of the two groups has statistically significant difference ($P<0.05$); the area of C group is significantly smaller than the area of group B. This also had a statistically significant difference ($P<0.05$) as shown in Tables 1, 2 and 3.

The corneal organization HE staining observed

The normal rabbit cornea is divided into 5 layers: the epithelium, lamina elastica, stroma, Descemet's layer and endodermis. The collagen fibers are tightly arranged,

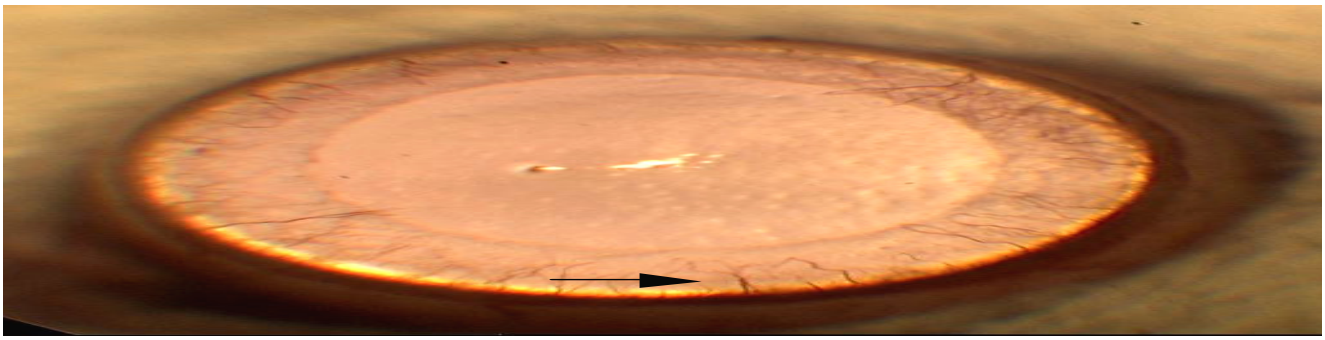

Figure 2. Group C CNV was significantly inhibited and thinning on 7 day.

Table 1. Area of corneal neovascularization in different groups (4 d) $\bar{x} \pm s$ mm/mm^2.

Indicator	A	B	C
L (The longest length of the CNV)	-	0.82±0.03	0.46±0.01
A (The area of CNV|)	-	5.35±0.16	3.25±0.09

P<0.05.

Table 2. Area of corneal neovascularization in different groups (7d) $\bar{x} \pm s$ mm/mm^2.

Indicator	A	B	C
L (The longest length of the CNV)	-	1.75±0.06	0.88±0.04
A (The area of CNV)	-	9.88±0.22	4.66±0.35

P<0.05.

Table 3. Area of corneal neovascularization in different groups (14 d) $\bar{x} \pm s$ mm/mm^2.

Indicator	A	B	C
L (The longest length of the CNV)	-	3.56±0.21	1.63±0.13
A (The area of CNV)	-	11.23±0.06	6.32±0.08

P<0.05.

without vascular structure. Neovascularization in the control group stroma have a large number of new blood vessels, in which the structure is loose and disordered, the lumen is large, and is infiltrated by inflammatory cell, and has a serious inflammatory response. In the Neovascularization intervention group, the corneal layer is seen to contain a smaller amount of inflammatory cells, sparser blood vessels, and smaller lumen (Figures 3 and 4).

The expressions of LP and VEGF in CNV were detected by immunohistochemical staining

Immunohistochemistry showed that the LP could be weakly expressed in the epithelium and stroma, the normal rat corneas VEGF lacked expression or minimally expressed only in the epithelial basement membrane, in the normal negative control group A. The expression of LP and VEGF is significantly increased in the control group B, and its main site is the region of the formation of new blood vessels and epithelium. Experimental intervention group C has sparser blood vessels, smaller lumen and the expression of LP and VEGF is significantly reduced (Figures 5, 6, 7 and 8).

DISCUSSION

The occurrence and development of CNV is a complex process, and VEGF plays a critical role. Tet is mainly present in the *Menispermaceae* woody perennial vine Tet

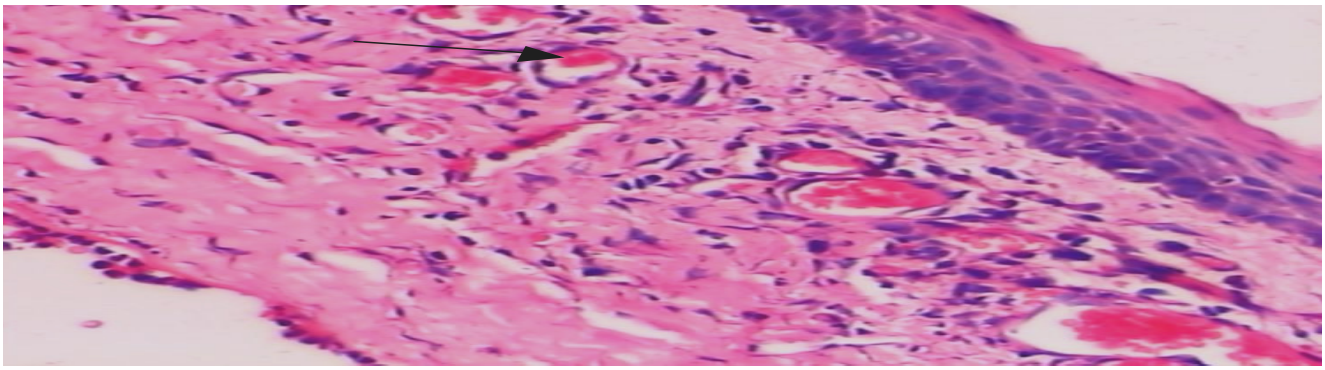

Figure 3. Control group, a large number of stroma neovascularization, loose structure disorders, inflammatory cell infiltration.

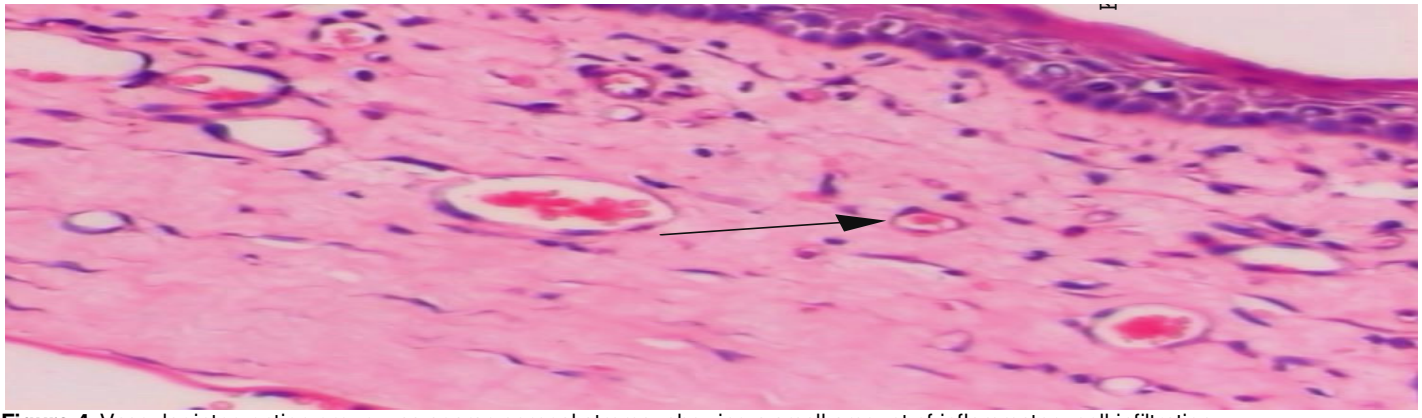

Figure 4. Vascular intervention group was sparse corneal stroma, showing a small amount of inflammatory cell infiltration.

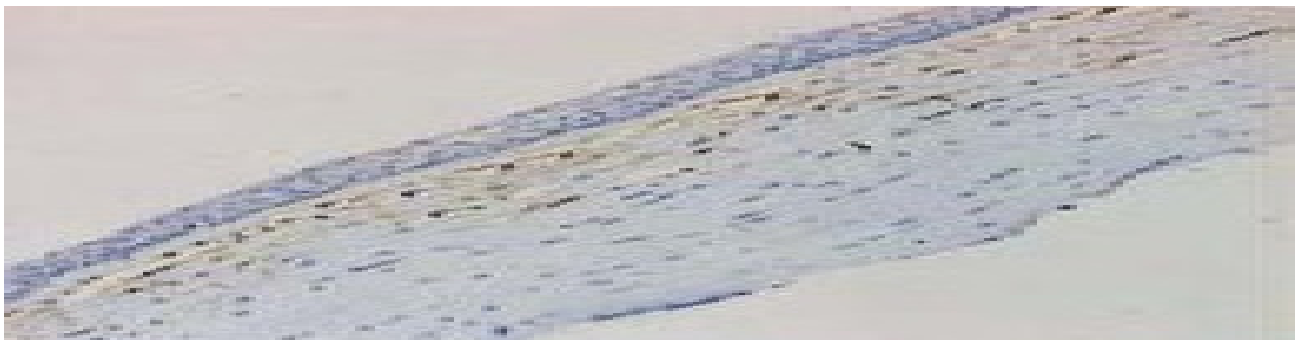

Figure 5. Control group B neovascularization LP was significantly increased in the cornea, mainly in the formation of new blood vessels on the cortical region and epithelium.

root, also named tetrandrine or tetrandrine Faso. Tet is the main active ingredient for Tet. Modern pharmacological research shows that Tet reduces pain, decreases blood pressure (Yu et al., 2004), and has anti-inflammatory (Wang et al., 2008) anti-silicosis (Sun et al., 2007) and anti-rheumatoid arthritis (Ho and Lai, 2004) properties. Tet is also a natural non-selective calcium channel resistant Lag agent, a calmodulin antagonist (Wang et al., 2005; Chiou et al., 2006) has a strong anti-tumorigenic (Chen, 2002) role. The main clinical role for Tet is the treatment of high blood pressure, silicosis, etc. A large number of studies have been reported about the pharmacological effects of Tet both at home and abroad, but there is little report about the treatment of corneal disease, and the mechanism is unclear. Some experiments showed that Tet could inhibit

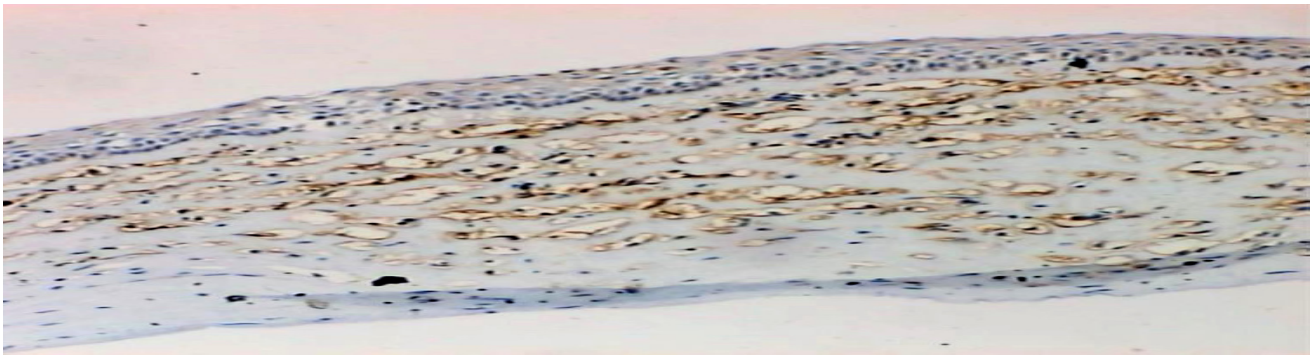

Figure 6. Control group B neovascularization VEGF was significantly increased in the cornea, mainly in the formation of new blood vessels on the cortical region and epithelium.

Figure 7. Experimental intervention group CLP was significantly lower.

Figure 8. Experimental intervention group C VEGF was significantly lower.

the experimental uveitis and keratitis rabbit. Some Animal vivisection experiments also showed that Tet could inhibit the rabbit posterior capsular opacification, intraocular lens membrane formation, and lipid peroxidation formation in the anterior chamber, posterior capsular opacification, intraocular lens membrane formation, and lipid peroxidation formation in the anterior chamber, Tet could mitigate the damage of eyes local tissue; *in vitro* studies have shown that It could inhibit the rabbit skin fibroblast and conjunctival fibroblast growth and proliferation significantly.

The results of this study show that Tet has some inhibitory effects on the rat's CNV. The mechanism may be related to the following

(1). To inhibits phospholipase A2 (PLA 2), to reduce lipid peroxidation and the generation of oxygen free radicals. Experiments prove Tet can act as a dose-dependent

inhibitor of PLA 2 activity (Wu and Ng, 2007). The inhibition of PLA2 can reduce lipid peroxidation and the generation of oxygen free radicals, also cause PGE2, LTB4 and PAF, and other vasoactive substances to reduce the generation that are conducive to the Tet Treatment of CNV.

(2). To prevent extracellular Ca^{2+} to influx. At present, the calcium overload of cytoplasm and mitochondrial can trigger lipid oxidative damage; lipid peroxidation can have an effect on the membrane structure, which can also promote the cytoplasm and mitochondria calcium overload. As a result, a vicious circle takes shape. Tet can be able to reduce the calcium concentration of neovascular cells, which weakened oxygen free radicals increase promoted by intracellular calcium overload.

(3). To inhibit the synthesis and release of inflammatory mediators and to reduce the generation of oxygen free radicals. The damage of PLA2 and other inflammatory mediators and vascular endometrial cells could be activated platelets; the release of oxygen free radicals could damage the cornea. Experiments prove (Vanathi et al., 2005). Tet not only inhibits the synthesis of PGE2, but also inhibits the synthesis of LTB4; and having a strong inhibition on monocytes, macrophages producing IL-1 and TNF-β cells also; also inhibit generation of PAF. By reducing the synthesis and release of the PG, LTB and IL-induced inflammation, Tet could reduce the generation of oxygen free radicals. Therefore it has the role of anti-oxidant activity. The studies have shown that Tet can significantly reduce the expression of VEGF and LP in CNV after a rat corneal alkali burn that can be used as a supplementary method for the treatment of CNV. The study showed that the expression VEGF and LP positive relevance, LP and VEGF have co-effect on regulating in the process of angiogenesis after the corneal alkali burn. To a certain extent, LP played an important role in the formation and development of CNV.

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