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Original Research Article

Inhibition of Corneal Neovascularization by Hydrazinocurcumin

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Abstract

Purpose: *To investigate the effect of hydrazinocurcumin on a human vascular endothelial growth factor (VEGF)-induced corneal neovascularization in rabbit model.*
Methods: *Murine corneal neovascularization (CorNV) was induced by two intrastromal implantations of VEGF polymer 2 mm from the limbus. Hydrazinocurcumin was administered topically on the cornea 4 times daily for 7 days. The therapeutic effects of hydrazinocurcumin were evaluated daily using slit-lamp. At the end of the treatment, the corneas were harvested for H&E staining, masson trichrome staining, immuno-histochemical study, and semi-quantification reverse transcription polymerase chain reaction (RT-PCR) was utilized for measurement of inflammation-related molecules.*
Results: *Topical application of hydrazinocurcumin had significant therapeutic effects on CorNV Hydrazinocurcumin extract treatment was more effective in suppressing CorNV in terms of vessel length and levels of cluster of differentiation 31 (CD31) proteins or angiogenesis-related genes such as VEGF, matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9). The average length of vessels in hydrazinocurcumin-treated group was only 17 % of that in the control group. Hydrazinocurcumin also inhibited inflammation more markedly by more effectively inhibiting mononuclear and polymorphonuclear cell infiltration into the corneal stroma and reducing levels of stromal cell-derived factor-1 (SDF1), tumor necrosis factor-alpha (TNF-α) and macrophage inflammatory protein-3 (MIP3a). In addition, the corneas of hydrazinocurcumin group had a more regular and compact architecture of collagen with thinner corneal thickness than those of the untreated group.*
Conclusion: *Hydrazinocurcumin inhibited human vascular endothelial growth factor (VEGF)-induced rabbit corneal neovascularization and thus can potentially be used for its treatment.*

Keywords: *Hydrazinocurcumin, Corneal neovascularization, Inflammation, Vascular endothelial growth factor, Corneal thickness*

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INTRODUCTION

Corneal neovascularization is found in 4.1 % of patients visiting general ophthalmology sections in the United States of America [1]. On penetration into the keratoplasty (PK) its treatment becomes dim [2]. Angiogenesis, the process of new blood vessel formation by endothelial cells involve membrane degradation by proteolytic enzymes, chemotactic migration toward the stimulus, proliferation of these cells, and formation of vascular loops [3,4]. Angiogenesis has a crucial role for the growth and penetration of cancer cells. Therefore, many angiogenesis inhibitors of natural product origin have been developed by various research groups [5-7].

Curcumin was isolated from the rhizome of Curcuma longa and is a promising chemopreventive agent presently in phase I clinical trials [8]. Curcumin exhibits anticancer activities against range of cancers including skin, fore stomach, duodenal, and colon carcinogenesis [9-12]. The anti-carcinogenic activity of curcumin is believed to be due to the inhibition of angiogenesis [13,14]. Study of structure-activity relationship for curcumin has led to the development of some more potent angiogenesis inhibitors like demethoxycurcumin (DC) and tetrahydrocurcumin (THC) [15]. It was demonstrated that aromatic ring of the phenol in curcuminoids and diketone moiety of THC play a vital role in the anti-oxidant activity [16,17]. Modification of the phenolic hydroxyl or methoxy groups resulted in the development of some analogs with potent activity as Phase 2 detoxification enzymes and the inhibition of HIV-1 integrase [18,19]. These findings revealed the importance of diketone moiety of curcumin in broad-range of biological activities.

In the present study, the anti-angiogenic activity of the hydrazinocurcumin (HC), an analog of the curcumin was studied. Several angiogenesis assays including endothelial cell proliferation, chemo invasion, capillary tube formation, and *in vivo* angiogenesis of chorioallantoic membrane of chick embryo were used for this study.

EXPERIMENTAL

Drug

Hydrazinocurcumin was obtained from Sigma (St Louis, MO, USA).

Corneal neovascularization induction and treatment strategy

All animal experiments were carried out following the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved (ref. no: 109/2014) by the Committee on the Ethics of Animal Experiments of the Affiliated Hospital of Academy of Military Medical Sciences, Shandong, China.

Eight-week old female mice (Chengdu Dashuo Biological Technology Co., Ltd., Chengdu, China) were maintained according to the guidelines of the National Institute of Health and Academy of Military Medical Sciences for the Care and Use of Laboratory Animals. Under general anaesthesia with intraperitoneal ketamine and chlorpromazine, corneal neovascularization was induced through two intrastromal implantations of VEGF polymer 2 mm from the limbus. Hydrazinocurcumin was administered topically on the corneas 4 times daily for 7 days. After completion of the treatment a slit lamp was used to take photograph and quantify CorNV using a method for determining corneal angiogenesis [17]. For additional studies the mice were sacrificed and their eyes were harvested.

Histological studies

For examination of histopathologic changes eyeballs were fixed with formalin and embedded with paraffin. The sections (3 µm) were analysed using haematoxylin and eosin and Masson's trichrome stain. The eyeballs were snap-frozen in optimal cutting temperature (OCT, Sakura Fine technical, Tokyo, Japan) for immuno-histochemical assay (IHC). Thin sections (5 µm) were fixed in ice-cold acetone for 10 min and subjected to conventional IHC protocols. The primary antibodies used were PE-conjugated anti-CD31 mAb (BD Pharmingen, CA, USA) mouse anti-SDF1 mAb (Santa Cruz, CA, USA). The secondary antibody used for SDF1 staining was rhodamine-conjugated goat anti-mouse IgG (Santa Cruz, CA, USA). The DAPI counterstained sections were examined using an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan).

Semi quantitative reverse transcription PCR

The total RNA from deep frozen mouse corneas was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Germany). The PrimeScript RT Reagent Kit (Takara, Japan) was used to reverse transcribe first strand cDNA from the 1 µg RNA. The primers for the genes of interest were subjected to PCR amplification. For PCR amplification denaturation was performed for 2 minutes at 95 °C, followed by 35 cycles of 1 min at 95 °C, 30 s at 60 °C and 1 min at 72 °C with an additional extension for 10 min at 72 °C at the end. PCR products were resolved in 1.5 % agarose gel, stained with ethidium bromide, and photographed with a high-resolution digital camera under UV illumination.

Statistical analysis

All the data expressed are mean ± SD (n = 3). One way analysis of variance and SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) were used for the analysis of differences in data. Differences were considered significant statistically at $p < 0.05$.

RESULTS

In vivo inhibitory effects of hydrazinocurcuminon CorNV

The corneas of both the control and the treatment group of mice were examined carefully for penetration of the vessels from limbus. The results showed penetration of vessels into the central cornea in case of the animals of control group by day 7 after induction of corneal neovascularization. However, topical administration of hydrazinocurcumin for 7 days, caused a significant inhibition of the development of corneal neovascularizationin the animals of the treatment group (Figure 1). The results from quantification statistics revealed that the average length of vessels in hydrazinocurcumin treated group was only 17 % of that in the control group.

Histological observation

H & E staining of the corneas revealed the presence of vessels along with the infiltration of mononuclear and polymorphonuclear cells in the stromal layer in the mice of control group. On the other hand, the corneas of hydrazinocurcumin treated mice were found to contain very few new vessels. In addition, no infiltrating mononuclear and polymorphonuclear cells were observed (Figure 2). The results from masson trichrome staining revealed the presence of dispersed stroma layers and fluffier collagen fibers in the animals of the control group. However, the dispersed stroma layers and fluffier collagen fibers were absent in the hydrazinocurcumin treated mice (Figure 2).

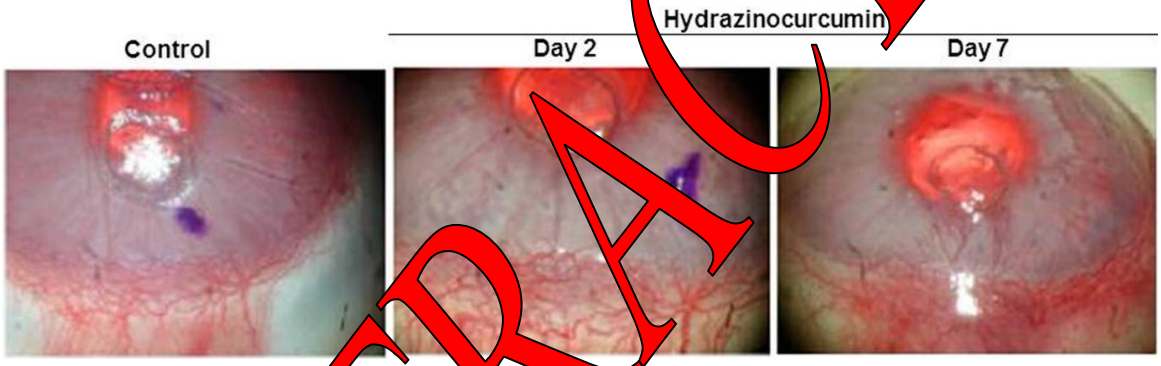

Figure 1: Macroscopic observation of corneal neovascularisation

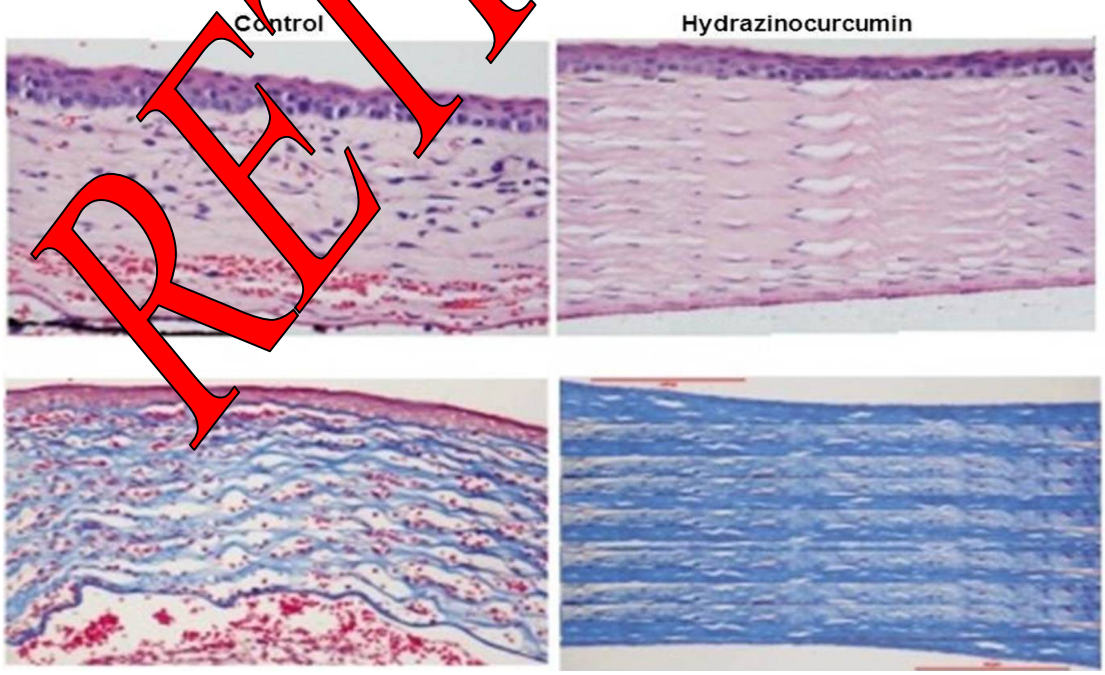

Figure 2: Histology of chemically burned corneas

Immunohistochemitry for CD31 and SDF1

The control group of mice showed a significant immunoreactivity for CD31, suggesting the presence of large number of vessels in the corneas. However, the corneas of the hydrazinocurcumin treated mice exhibited very small immunoreactivity for CD31 (Figure 3). The lymphocyte chemoattractant cytokine, SDF-1 is known to be expressed significantly in the fibroblasts in cornea. However, the expression of SDF1 in the corneas of the animals treated with hydrazinocurcumin was found to be significantly lower than those of the control group (Figure 3).

Inhibitory effects of hydrazinocurcumin on the expression of genes associated with angiogenesis or inflammation in burned cornea

mRNA level of angiogenesis-related factors including VEGF, MMP2 and MMP9 was significantly decreased compared to the animals of the untreated group. Similarly, the level of inflammation-related factors including SDF1, TNFα and MIP3a was also decreased significantly in hydrazinocurcumin treated mice compared to untreated group (Figure 4).

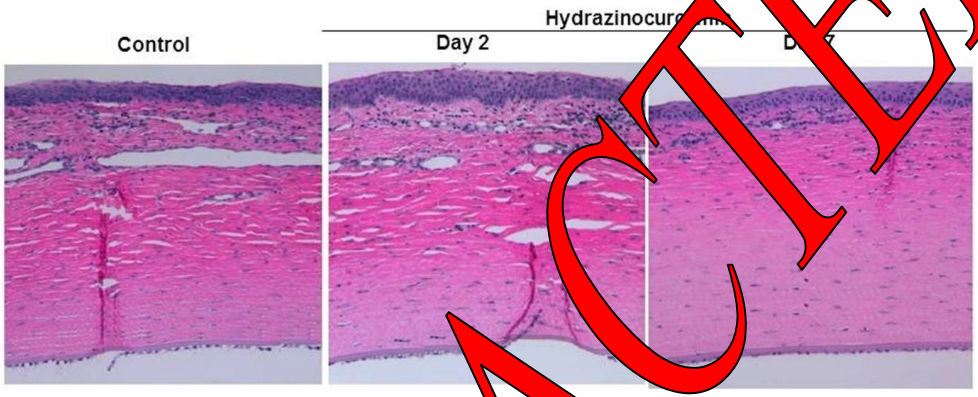

Figure 3: CD31and SDF1 expression profile in alkali burned mouse cornea

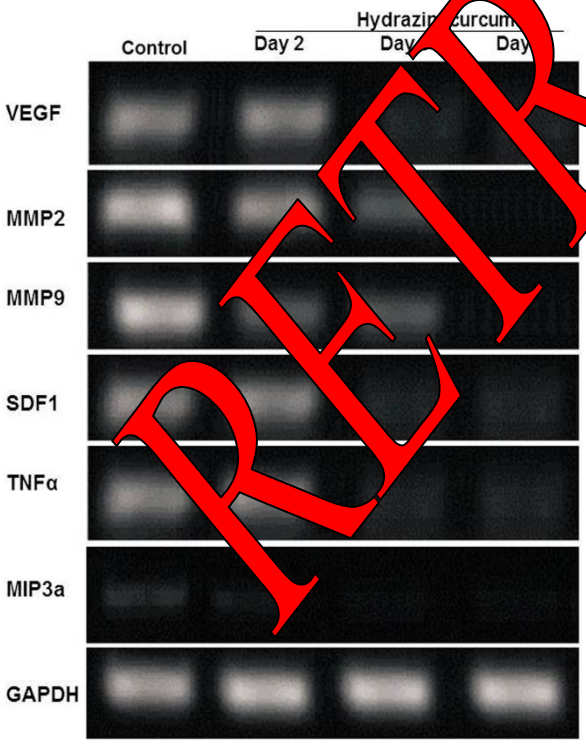

Figure 4: Changes in angiogenesis- and inflammation-related genes in hydrazinocurcumin-treated or un-treated corneal neovascularization corneas

DISCUSSION

In order to maintain transparency for the purpose of refraction and transmission of light the cornea is an avascular tissue. However, infectious and inflammatory processes are reported to induce the growth of new vessels leading to corneal neovascularisation and finally scarring, edema, and blindness [3]. It is reported that corneal neovascularization occurs through the imbalance of angiogenic and anti-angiogenic protein factors. In vascularized human corneas a significant up-regulation of vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP), and basic fibroblastic growth factor (bFGF) is observed [5].

In corneal neovascularization ocular surface develops inflammatory disorders therefore the control or prevention of inflammation may be employed for its treatment. It is reported that corneal neovascularization induced through two intrastromal implantations of VEGF polymer is associated with corneal inflammation and resembles inflammatory corneal diseases [20,21]. Therefore, corneal neovascularisation induced through two intrastromal implantations of VEGF polymer serves as a suitable animal model for the study of inflammatory corneal

neovascularization. There are a number of natural products known to possess anti-angiogenic properties which include genistein [22], shark cartilage [23], curcumin [24,25], propolis extract [26] and AME [14-17].

The present study demonstrates that hydrazinocurcumin inhibits human vascular endothelial growth factor (VEGF)-induced rabbit corneal inflammation and corneal neovascularization significantly. Thus hydrazinocurcumin may be used for the treatment of corneal diseases involving neovascularization and inflammation. Hydrazinocurcumin was more effective in inhibiting corneal neovascularization at all levels, namely gross appearance, histological or cellular levels, and molecular levels.

CONCLUSION

The results show that hydrazinocurcumin significantly inhibits inflammation and angiogenesis, and therefore, may have promise for the treatment of corneal neovascularization.

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