PSH–ES inhibits corneal neovascularization

**Inhibitory effect of polysulfated heparin endostatin on alkali burn induced corneal neovascularization in rabbits**

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**Abstract**

• **AIM:** To investigate anti–angiogenic effects of polysulfated heparin endostatin (PSH–ES) on alkali burn induced corneal neovascularization (NV) in rabbits.

• **METHODS:** An alkali burn was made on rabbit corneas to induce corneal NV in the right eye of 24 rabbits. One day after burn creation, a 0.2 mL subconjunctival injection of 50 μg/mL PSH–ES, 50 μg/mL recombinant endostatin (ES), or normal saline was administered every other day for a total of 14d (7 injections). Histology and immunohistochemistry were used to examine corneas. Corneal NV growth was evaluated as microvessel quantity and corneal vascular endothelial growth factor (VEGF) expression was measured by immunohistochemical assay.

• **RESULTS:** Subconjunctival injection of ES and PSH–ES resulted in significant corneal NV suppression, but PSH–ES had a more powerful anti–angiogenic effect than ES. Mean VEGF concentration in PSH–ES treated corneas was significantly lower than in ES treated and saline treated corneas. Histological examination showed that corneas treated with either PSH–ES or ES had significantly fewer microvessels than eyes treated with saline. Additionally corneas treated with PSH–ES had significantly fewer microvessels than corneas treated with ES.

• **CONCLUSION:** Both PSH–ES and recombinant ES effectively inhibit corneal NV induced by alkali burn. However, PSH–ES is a more powerful anti–angiogenic agent than ES. This research has the potential to provide a new treatment option for preventing and treating corneal NV.

• **KEYWORDS:** polysulfated heparin endostatin; corneal neovascularization; chemical burns; rabbits

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**INTRODUCTION**

The normal cornea is transparent and free of blood vessels. Neovascularization (NV) plays an important role in the pathogenesis of corneal disorders. When the balance between angiogenic and anti-angiogenic factors becomes imbalanced towards angiogenesis, corneal NV often occurs. Many corneal diseases, including chemical burns, thermal burns, infection, and immunologic diseases are complicated by corneal NV. Some treatment modalities, including laser photocoagulation [1], medication [2,3], and surgery [4], are used to treat corneal neovascular diseases. Unfortunately, these treatments have many limitations and associated complications. Therefore, effective medications to treat corneal NV are greatly needed.

Endostatin (ES), an endogenous inhibitor of endothelial cell proliferation and angiogenesis, specifically inhibits vegetative vascular endothelial cell growth [5]. Therefore, ES effectively inhibits angiogenesis and tumor growth. Over the past few years, ES has drawn some attention for its ability to treat corneal, retinal, and choroidal NV in the experimental setting. However, there are many obstacles to its use in the clinical setting, including the high maintenance dose required to maintain its efficacy, its poor chemical stability, its short half-life, and other shortcomings related to protein drugs. Therefore, chemical modification has been widely studied in an effort to improve efficacy and to overcome some of the inherent disadvantages of protein
drugs\textsuperscript{[10,11]}. In a previous study, ES was successfully modified with polysulfated heparin (PSH) \textsuperscript{[12]}. These encouraging results made us curious about the effects of PSH modified ES (PSH-ES) on angiogenesis. Here, a rabbit model of corneal NV was used to examine and compare ES and PSH-ES bioactivity. A corneal NV assay and corneal vascular endothelial growth factor (VEGF) expression were used to quantify anti-angiogenic activity of the two compounds.

**MATERIALS AND METHODS**

This study was carried out with the assistance of the Institute of Biochemical and Biotechnological Drugs at the Shandong University School of Pharmaceutical Science. Our methods of evaluating corneal NV are based on methods widely used in previous studies to measure anti-angiogenic activity of ES \textit{in vivo} \textsuperscript{[10]}. Twenty-four 6mo old New Zealand albino rabbits of either sex purchased from the Shandong University Laboratory Animal Department were used in this experimental study. Animals were treated in accordance with the Shandong University Animal Experimentation Ethic Committee (AECE) guidelines. The study protocol was approved by the AECE. All animal care, use, and treatment were in strict agreement with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research. Both ES and PSH-ES were kindly provided by the Institute of Biochemical and Biotechnological Drugs at the Shandong University School of Pharmaceutical Science (Jinan, Shandong Province, China). The preparation of both compounds has been previously described\textsuperscript{[12,14]}. Ready-to-use streptavidin-peroxidase (SP) immunohistochemical reagent boxes were purchased from the Zhongshan Chuyangi Biochemical Company (Guangdong, Guangdong Province, China). All other chemicals were commercially obtained and were of the highest purity available. Therefore, they were used without further purification.

**Alkali-induced Corneal Neovascularization** Before creating corneal alkali burns, rabbits were examined for ocular abnormalities. Once deemed healthy, rabbits were randomly divided into three groups of eight animals each. Animals were anesthetized using a combination of ketamine hydrochloride (25 mg/kg) and chlorpromazine (25 mg/kg), administered intramuscularly. Once the proper level of anesthesia had been attained, as determined with corneal response, central cornea of the right eye was burned. This was done by placing a NaOH-soaked (1 mol/L) circular piece of filter paper (6 mm in diameter) on the corneal surface for 60s. After the filter paper was removed, the ocular surface and the conjunctival sac were immediately rinsed with 20 mL of a balanced salt solution for 1min. One day after burn creation, 0.2 mL of PSH-ES (50 μg/mL), ES (50 μg/mL), or physiological saline was injected into the subconjunctival tissues. Animals received repeated injections one time every other day for a total of 14d (7 injections).

After each injection, topical antibiotic ointment was administered to the burned eye to minimize the risk of post-injection infection.

**Evaluation of Corneal Neovascularization and Inflammation** The same observer carefully quantified corneal NV with a slit lamp each day after the first injection. At the same time, the ocular surface was also examined for corneal ulceration and bulbar conjunctival hyperemia and edema. Eyes were photographed using a digital camera from day 1 to day 16. The images taken on days 4, 7, 10, and 13 were selected and transferred to a computer for further analysis. Using these photographs, vessel growth from the corneoscleral limbus into the clear cornea was automatically quantified as vessel area (in mm\(^2\)) by Image-Pro Plus software (MediaCybernetics, Inc., Rockville, MD, USA)\textsuperscript{[15–17]}.

**Histology and Immunohistochemistry** Rabbits were sacrificed with an overdose of intravenous pentobarbital sodium 16d after alkali corneal burn creation. Eyes were then enucleated and each cornea, including the adjacent 2 mm of scleral tissue, was removed and quickly fixed in formalin. After 24h, tissue specimens were dehydrated, infiltrated, embedded in paraffin, and sectioned with a microtome. Sections were then stained with hematoxylin and eosin and corneal microvascular density was determined by highlighting vessels. This was done with anti-CD34 antigen monoclonal antibody immunostaining, according to manufacturer instructions (1:200 dilution, 2h room temperature incubation). Cell staining results were given a grade of 1 (yellow), 2 (brown), or 3 (sepia), based on cytoplasm color. The LUZEX-F (Japan) image analyzer applied staining results to obtain VEGF levels, as indicated by grey-scale levels. Microvascular densities were obtained using previously established methods\textsuperscript{[18]}. Three cut specimens were selected for each eye. New corneal vessels were counted in the 5 areas of highest vascular density at a magnification of 400×. Microvascular density was expressed as the mean number of vessels per field of view area.

**Statistical Analysis** Statistical analyses were performed using SPSS statistical software (version 15.0, SPSS, Chicago, IL, USA). The statistical significance of differences between the PSH-ES, ES, and control groups was determined using one-way analysis of variance (ANOVA) and unpaired Student's \(t\)-tests. Data are reported as mean± standard deviation and statistical significance was defined as \(P<0.05\).

**RESULTS** Rabbit eyes were examined and photographed under a surgical microscope each day following corneal alkali burn creation. Corneal opacity and angiogenesis gradually increased in all three groups. Only slight edema and a tiny, slowly growing area of focal corneal NV was noted at the limbus in eyes treated with PSH-ES (Figure 1A). In eyes
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**Figure 1** Corneal neovascularization in alkali–burned rabbit corneas. Efficacy comparison of treatment with PSH–ES and ES after chemical corneal burn

Thirteen days following alkali burn, PSH-ES treated eyes (A) had significantly less neovascular growth than ES treated eyes (B). Eyes treated with PSH-ES (A) or ES (B) had significantly less neovascular growth than control (C) eyes.

**Figure 2** Expression of vascular endothelial growth factor (VEGF) in corneal tissue, as detected by immunohistochemistry. All measurements were taken 16d following alkali burn

Minute levels of VEGF were detected in the PSH-ES group (A), low levels of VEGF were detected in the ES group (B), and high levels of VEGF were detected in the control group (C).

**Table 1** Microvascular length and corneal neovascularization in each study group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 13</th>
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<tbody>
<tr>
<td>PSH-ES</td>
<td>0.42±0.12</td>
<td>1.38±0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.15±0.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.32±1.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ES</td>
<td>0.88±0.21</td>
<td>1.73±0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68±1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.49±1.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>1.35±0.86</td>
<td>2.38±0.56</td>
<td>3.92±1.29</td>
<td>5.85±1.28</td>
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<sup>b</sup>Statistically different than the control group (P<0.05). ES: Endostatin; PSH-ES: Polysulfated heparin endostatin.

We theorized that the effect of PSH-ES on corneal NV was through down regulation of VEGF, a known mediator of corneal NV and inflammation. Therefore, we used immunohistochemistry to measure corneal VEGF levels. In the treatment groups, rabbit eyes treated with PSH-ES and ES displayed significantly weaker grade 1 and grade 2 VEGF signals, respectively, in the same area under the same exposure intensity (Table 2; Figure 2A, 2B). In contrast, in the control group, immunohistochemistry assay showed an intensive, grade 3 VEGF signal in stromal and intravascular inflammatory cells (Figure 2C).

Morphologically, only a small number of new corneal vessels were detected in eyes treated with PSH-ES or ES. Additionally, the number of microvessels seen on day 16 was significantly lower in the PSH-ES group than in the ES group (Table 2; Figure 3A, 3B, P<0.011). In contrast, large numbers of new corneal vessels, observed throughout the entire stroma, were detected in control eyes (Figure 3C).
DISCUSSION

Corneal NV has been shown to reduce visual acuity[19] and is often induced by infectious, inflammatory, degenerative, ischemic corneal disease, and ischemic limbal stem cell barrier breakdown. Many angiogenesis inhibitors have been studied in an effort to find a new medical treatment for corneal NV. These include angiostatin [20], thalidomide [21], prolactin[22], somatostatin[23], and ES[6].

Recent studies have shown that recombinant ES inhibits corneal NV and tumor neovascular growth by suppressing VEGF expression [24,25]. ES, a protein fragment originating from type XVIII collagen, is an endogenous angiogenesis inhibitor and an antitumor factor[29]. The State Food and Drug Administration (SFDA) in China approved the use of ES in patients with non-small cell lung cancer some time ago (September 2005)[27]. However, its short half-life, required high doses, and poor stability have hindered its utilization [8,29]. In a previous study, ES was successfully modified with polysulfated heparin, after which it was purified by column chromatography [12]. In the current study, we compared anti-angiogenic property difference between ES and PSH-ES in an experimental model of corneal NV. The CNV assay results revealed that the PSH-ES corneal NV inhibitory effect was stronger than that of ES. Additionally, VEGF expression was significantly lower in eyes treated with PSH-ES than in those treated with ES. This indicates, for the first time, that modified PSH-ES has better anti-angiogenic activity than the original molecule. It is known that PSH-ES is a larger molecule than ES. In fact, the molecular weight of ES and the PSH modifier are 20 kD and 5.2 kD, respectively, as measured with gel permeation chromatography [12]. The SDS-PAGE technique also showed that the molecular weight of PSH-ES is 35 kD. Therefore, we presume that, on average, one ES molecule conjugates with three PSH molecules. In this previous study, a corneal NV assay demonstrated that, when modified by PSH, ES activity had a prolonged reaction time and purified PSH-ES had better heat stability than ES at 25°C and 37°C [12]. We conclude that these observations may underlie the stronger anti-angiogenic properties of PSH-ES over ES in vivo. More obviously, the greater amount of VEGF down-regulation observed in the presence of PSH-ES than in the presence of ES also contributed to the higher efficacy of PSH-ES in inhibiting NV. After modification, PSH-ES likely has a longer half-life than ES [29], which allows an adequate amount of substance to be present longer. The PSH-ES compound is structured so that the PSH is on the outside, protecting ES like a blanket and making it markedly more stable than native ES. Lastly, PSH also has anti-angiogenic properties, and PSH and ES may work synergistically[30].

Animal studies are currently underway to examine the immunogenicity and toxicity of ES when it is used alone and in combination with chemotherapeutic agents. After chemical modification by PSH, modified ES has a higher stability and bioactivity than native ES. Polysulfated heparin has been widely used as chemical modifier proteins and peptides because it is not bioactive and has no known related adverse effects. There are currently many modifiers without bioactivity to choose from, but we chose PSH, a functional polysaccharide, as the ES modifier because the modification

Table 2 Quantified levels of vascular endothelial growth factor (VEGF) and the number of microvessels at day 16

<table>
<thead>
<tr>
<th>Groups</th>
<th>VEGF Levels (gray scale)</th>
<th>Microvessels (n)</th>
</tr>
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<tbody>
<tr>
<td>PSH-ES</td>
<td>101.32±17.46b</td>
<td>1.04±0.82b</td>
</tr>
<tr>
<td>ES</td>
<td>123.56±20.13b</td>
<td>3.59±1.75b</td>
</tr>
<tr>
<td>Saline</td>
<td>153.15±24.54</td>
<td>6.32±2.75</td>
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</tbody>
</table>

Microvessels were counted under 400X magnification. bStatistically different than controls. PSH-ES: Polysulfated heparin endostatin; ES: Endostatin.
results in better bioactivity and stability of ES. As in previous reports, we observed no adverse effects directly related to PSH in this study.

In summary, this is the first study to examine the anti-angiogenic properties of PSH-ES a corneal NV model. Our experiments demonstrated that, with only a small change in the secondary structure [12], PSH-ES has better heat stability, greater anti-angiogenic activity, and larger VEGF down-regulation than ES. Therefore, PSH-ES may be an ideal candidate for preclinical and, eventually, clinical development for the treatment of corneal NV.

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Conflicts of Interest: Li ZN, None; Yuan ZF, None; Mu QY, None; Hu M, None; Cao LJ, None; Zhang YL, None; Liu L, None; Ge MX, None.

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