The effects of deproteinized calf blood extract (DCBE) on the pathophysiological changes of the ocular surfaces in mice with xerophthalmia

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Abstract: Dry eye is a common clinical disease, and its incidence rate is increasing. Therefore, it is important to investigate new methods for the treatment of dry eye. A C57BL/6 mouse dry eye model was induced using benzalkonium chloride. The mice were treated with different concentrations of deproteinized calf blood extractives (DCBE) (0, 20%, 50%, and 100%) 3 times/day for 28 days. The tear fluid, cornea fluorescein staining, and corneal inflammation index were measured at 0, 3, 7, 14, and 28 days after the treatment. The number of goblet cells was analyzed. The TNF-α, IL-1β, ICAM-1, p-p38 MAPK, and K10 expressions in the orbital tissue were tested. The tear fluid and number of goblet cells were significantly reduced in the experimental group compared with the negative control (P < 0.05). The tear fluid was significantly increased, but the cornea fluorescein staining and the corneal inflammation index significantly declined starting on the 7th day after the DCBE intervention compared with the positive control (P < 0.05). The number of goblet cells was elevated, but the TNF-α, IL-1β, ICAM-1, p-p38 MAPK, and K10 expressions were downregulated in the DCBE groups compared with the positive control in a dose dependent manner (P < 0.05). DCBE exhibited treatment efficacy in mouse dry eye, which is induced using benzalkonium chloride by blocking the ocular surface squamous metaplasia and alleviating inflammation.

Keywords: Deproteinized calf blood extractives, benzalkonium chloride, dry eye, inflammation

Introduction

Dry eye is a type of ocular surface disease which is characterized by ocular surface pathological changes caused by various factors. It may induce tear film instability, changes of tear quality and quantity, and inflammation, leading to eye discomfort and visual impairment that seriously affects one’s daily activities and quality of life [1]. Its incidence exhibits a rising trend resulting from the use of electronic products and the aggravation of environmental contamination in recent years [2]. Mild dry eye generally can be alleviated by improving one’s lifestyle and living environment, but moderate or severe cases usually require long-term treatment. Inappropriate or delayed treatment may aggravate the illness, causing corneal ulcers and epithelial squamous metaplasia, and even leading to blindness [3]. The treatment methods mainly include drug therapy, physical therapy, and surgery. Drug therapy is mostly used, including anti-inflammatory drugs, promoting tear secretion, artificial tears, and autologous serum, etc. However, all of these treatment approaches have their own disadvantages [4, 5]. Anti-inflammatory drugs often have side-effects and cannot be used over a long period. Drugs promoting tear secretion only relieve the symptoms and exhibit a poor curative effect in moderate to severe dry eye. Artificial tears can only lubricate the ocular surface, while its long-term application may damage the corneal epithelium without any clear curative effect. Autologous serum is rich in vitamin A, fibronectin, and growth factors and has been reported to have a significant effect on treating moderate to severe dry eye with fewer side effects [6, 7]. However the preparation and preservation of autologous serum is difficult, and the serum brings a risk of
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Anemia and infection. Thus, searching for a drug with high treatment efficacy and few side effects is of great significance in the clinical treatment of dry eye.

Deproteinized calf blood extractives (DCBE) is a type of blood extractive rich in amino acids, sugar, fat, low molecular peptide, and nucleic acid. DCBE is a type of growth factor that increases the mitochondrial application of oxygen and glucose, prompts adenosine triphosphate (ATP) synthesis, accelerates metabolism, activates a variety of enzyme activities, improves tissue hypoxia, facilitates tissue damage repair, and extends cell survival [8, 9]. DCBE is widely applied in the treatment of multiple diseases, such as traumatic brain injury, cerebral infarction, and refractory wounds [8, 10, 11]. However, its role in the treatment of moderate to severe dry eye has not been fully elucidated. This study adopted a mouse dry eye model which was established by benzalkonium chloride to explore the efficacy and mechanisms of DCBE, aiming to provide a basis for the clinical application of DCBE in the treatment of dry eye.

Materials and methods

Mouse dry eye modeling

A total of 30 healthy male C57BL/6 mice (the mice were provided by Kunming Medical University) aged 6-8 weeks were raised in a standard environment at a temperature of 18-22°C and a relative humidity of 50-60%. Twenty-four mice were used to establish the dry eye model. The mice were treated with 0.2% benzalkonium chloride eye drops at 5 μl three times/day for 7 days. Tear fluid, cornea fluorescein staining, and the corneal inflammation index were used to evaluate the establishment of the model as described previously [12].

The mice were used for all the experiments, and all the procedures were approved by the Animal Ethics Committee of the second people’s Hospital of Yunnan Province.

Mice grouping

The 24 dry eye mice were randomly divided into different concentrations of DCBE (0, 20%, 50%, and 100%) (weight/volume in PBS) groups with six in each group. The remaining six mice were treated as a negative control. The DCBE were provided by the Sinqi Pharmaceutical Co., Ltd (Shenyang, China). The mice were treated with DCBE through an intraperitoneal injection 3 times/day for 28 days.

Index detection

Schirmer I test: Schirmer I tests were performed using phenol red thread (Yokota, Japan) at 0, 3, 7, 14, and 28 days after the intervention. The mice received general anesthesia, and the thread was put on the inferior palpebral conjunctiva for 15 s. The length of red on the thread was recorded.

Cornea fluorescein staining: The mouse eye was dropped with 1% sodium fluorescein at 0, 3, 7, 14, and 28 days after the intervention. The staining was observed after 1 min under the cobalt-blue light of a slit lamp microscope. The results were scored according to the Park integral method. 0 points refers to no color, 1 point refers to dot color, 2 points refers to dot color ≤ 1/4 quadrant, 3 points refers to dot color between 1/4-1/2 quadrant, and 4 points refers to dot color ≥ 1/2 quadrant.

Corneal inflammation index: The corneal inflammation index was evaluated using a slit lamp microscope at 0, 3, 7, 14, and 28 days after the intervention. The inflammation index was composed of three parts, including ciliary congestion (0 points, no congestion; 1 point, congestion band < 1 mm; 3 points, congestion band > 2 mm), central corneal edema (0 points, no edema; 1 point, slight edema with a clear iris texture, 2 points, moderate edema with an unclear iris texture; 3 points, invisible iris and pupil), and peripheral corneal edema (same as central corneal edema). Corneal inflammation index = total score of three parts/9.

Goblet cell counting: A few small eye wall fragments were dissected and fixed in 10% formalin and embedded in wax. The section of eye tissue containing the integral eyelid and palpebral conjunctival was stained with PAS. Conjunctiva goblet cells were stained deep red in the cytoplasms. Six sections at different parts were selected for counting in each eye to calculate the number of goblet cells.

Western blot

The tissue was lysed using a RIPA lysis buffer at 1:5 on ice for 30 min. After being centrifuged at
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14000 rpm and 4°C for 30 min, the supernatant was stored at -80°C. The protein was quantified using the BCA method and separated on 12% SDS-PAGE. After electronic transfer, the membrane was incubated with primary antibodies (TNF-α, 1:1000; IL-1β, 1:500; ICAM-1, 1:500; p-p38 MAPK, 1:500; K10, 1:1000; β-actin, 1:2000, Abcam) at 4°C overnight. After they were washed with PBST, the membranes were incubated with a secondary antibody (1:5000, Genetech) at room temperature for 30 min followed by a wash with PBST, and the addition of ECL for 1 min and subsequent exposure and development.

Statistical analysis

SPSS 18.0 software was used for the data analysis. The measurement data were presented as the means ± standard deviations and compared using a one-way ANOVA, SNK, or LSD-t test. P < 0.05 was considered statistically significant.

Results

Schirmer I test

The tear fluid was significantly reduced in the experimental group compared with the negative control (P < 0.05). The tear fluid was significantly increased on the 7th, 14th, and 28th days after the DCBE intervention compared with the positive control and pre-intervention (P < 0.05), but it showed no statistical changes on the first three days (P > 0.05) (Table 1).

Table 1. Schirmer I test

<table>
<thead>
<tr>
<th>Tear fluid (mm)</th>
<th>Intervention time (day)</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td></td>
<td>5.68±0.67</td>
<td>5.54±0.62</td>
<td>5.49±0.59</td>
<td>5.52±0.56</td>
<td>5.63±0.64</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>2.47±0.16a</td>
<td>2.44±0.17a</td>
<td>2.53±0.16a</td>
<td>2.62±0.18a</td>
<td>2.56±0.17a</td>
</tr>
<tr>
<td>20% DCBE</td>
<td></td>
<td>2.49±0.17a</td>
<td>2.56±0.19a</td>
<td>3.08±0.17ab,c</td>
<td>3.57±0.21ab,c</td>
<td>3.74±0.30ab,c</td>
</tr>
<tr>
<td>50% DCBE</td>
<td></td>
<td>2.45±0.18a</td>
<td>2.61±0.17a</td>
<td>3.54±0.24ab,c</td>
<td>3.88±0.28ab,c</td>
<td>4.12±0.41ab,c</td>
</tr>
<tr>
<td>100% DCBE</td>
<td></td>
<td>2.51±0.16a</td>
<td>2.67±0.18a</td>
<td>3.86±0.25ab,c</td>
<td>4.35±0.32ab,c</td>
<td>4.83±0.47ab,c</td>
</tr>
</tbody>
</table>

*aP < 0.05, compared with the negative control; bP < 0.05, compared with the positive control; cP < 0.05, compared with pre-intervention.

Table 2. Cornea fluorescein staining

<table>
<thead>
<tr>
<th>Cornea fluorescence staining</th>
<th>Intervention time (day)</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td></td>
<td>0.12±0.27</td>
<td>0.13±0.32</td>
<td>0.11±0.29</td>
<td>0.14±0.26</td>
<td>0.10±0.28</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>3.34±0.51a</td>
<td>3.36±0.49a</td>
<td>3.31±0.48a</td>
<td>3.24±0.47a</td>
<td>3.44±0.43a</td>
</tr>
<tr>
<td>20% DCBE</td>
<td></td>
<td>3.38±0.53a</td>
<td>3.27±0.46a</td>
<td>2.86±0.43ab,c</td>
<td>2.08±0.40ab,c</td>
<td>0.42±0.29ab,c</td>
</tr>
<tr>
<td>50% DCBE</td>
<td></td>
<td>3.33±0.48a</td>
<td>3.20±0.44a</td>
<td>2.42±0.41ab,c</td>
<td>1.05±0.36ab,c</td>
<td>0.34±0.32ab,c</td>
</tr>
<tr>
<td>100% DCBE</td>
<td></td>
<td>3.36±0.45a</td>
<td>2.98±0.51a</td>
<td>1.68±0.39ab,c</td>
<td>0.59±0.31ab,c</td>
<td>0.21±0.31ab,c</td>
</tr>
</tbody>
</table>

*aP < 0.05, compared with the negative control; bP < 0.05, compared with the positive control; cP < 0.05, compared with pre-intervention.

Cornea fluorescein staining

The cornea fluorescein staining score was significantly increased in the experimental group compared with the negative control (P < 0.05). The cornea fluorescein staining score was significantly declined on the 7th, 14th, and 28th days after the DCBE intervention compared with the positive control and pre-intervention (P < 0.05), but it showed no statistical changes on the first three days (P > 0.05) (Table 2).

Corneal inflammation index

The corneal inflammation index was significantly elevated in the experimental group compared with the negative control (P < 0.05). The corneal inflammation index was significantly reduced on the 7th, 14th, and 28th days after the DCBE intervention compared with the positive control and pre-intervention (P < 0.05), but it showed no statistical changes on the first three days (P > 0.05) (Table 3).
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The number of Goblet cells was significantly elevated in the DCBE groups compared with the positive control in a dose dependent manner (P < 0.05) (Figure 1).

K10 protein expression

K10 expression was significantly upregulated in experimental group compared with the negative control. It was gradually downregulated in the DCBE groups compared with the positive control in a dose dependent manner (P < 0.05) (Figure 2).

TNF-α, IL-1β, ICAM-1, and p-p38 MAPK protein expressions

The TNF-α, IL-1β, ICAM-1, and p-p38 MAPK levels were significantly enhanced in the experimental group compared with the negative control (P < 0.05). Their expressions were gradually weakened in the DCBE groups compared with the positive control in a dose dependent manner (P < 0.05) (Figure 3).

Discussion

Dry eye is a common ocular surface disease affected by corneal epithelium damage, reduced stability of the tear membrane, tear osmotic pressure elevation, and ocular surface inflammatory reactions [13]. The pathogenesis of dry eye is complex and has not been elucidated. In the clinic, dry eye is often accompanied by a different degree of ocular surface inflammation which is featured as conjunctival congestion [14]. Persistent inflammation damages the repair and defense of the ocular surface. A large amount of inflammatory cytokines will not only damage the nerve conduction of the normal lacrimal gland secretion, leading to tear membrane instability, but it will also damage the structure and function of the ocular surface and the lacrimal gland tissue [4, 15]. In this study, a mouse dry eye model exhibited de-
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Increased tear fluid, elevated cornea fluorescence staining, an enhanced inflammation index, and a reduced number of goblet cells, which coincides with the clinical symptoms [16]. It suggests that the successful construction of a mouse dry eye model was achieved [12].

This study adopted different concentrations of DCBE to treat dry eye in mice. It was found that tear fluid was significantly increased from the 7th day after the DCBE intervention compared with the positive control and pre-intervention. Its recovery was good on the 28th day. The cornea fluorescein staining score and the cornea inflammation index showed the opposite results compared with the tear fluid. In addition, the number of goblet cells was significantly elevated in the DCBE groups compared with the positive control with dose dependence, indicating the curative effect of DCBE on the mouse dry eye model which was induced by benzalkonium chloride. DCBE is rich in multiple bioactive factors, so it can improve the utilization of oxygen and glucose in tissue and provide energy for cell proliferation, division, and migration [8, 9]. Moreover, DCBE can promote cell injury repair by affecting the bioactivity of various enzymes. DCBE has exhibited a good curative effect on several types of tissue damage, such as cranio-cerebral injury, diabetic foot ulcer, corneal injury, and refractory trauma [8, 10, 11].

Ocular surface squamous metaplasia refers to the pathological process of the secretory nonkeratinized stratified epithelium transferring into the nonsecretory keratinized squamous epithelium. It may be related to ocular surface local persistent chronic inflammation [17]. Cytokeratin 10 (K10) is usually used to detect ocular surface squamous metaplasia [18]. In this study, a Western blot analysis found that K10 expression was significantly upregulated in the experimental group compared with the negative control. It indicated that ocular surface squamous metaplasia occurred in the mouse dry eye model. The K10 level was significantly reduced after DCBE intervention, revealing that DCBE effectively prevents and reverses squamous metaplasia. It may be related to the effect of lubricating the ocular surface, which can promote corneal epithelium repair and alleviate the inflammation reaction.

Ocular surface inflammation is one of the major causes of dry eye. Intercellular cell adhesion molecule-1 (ICAM-1) is an important adhesion molecule mediating the adhesion reaction [19], and it is also an indicator of ocular surface inflammation, which is positively correlated with dry eye damage [20, 21]. P38 mitogen-activated protein kinases (p38 MAPKs) are key kinases mediating cell stress and the inflammatory response. Activated p38 MAPKs (p-p38 MAPK) participates in the regulation of the inflammatory response and immune regulation by affecting various transcription factors, including TNF-α, IL-1β, and IL-6. TNF-α and IL-1β...
are important inflammatory cytokines involved in the pathogenesis of dry eye. This study tested TNF-α, IL-1β, ICAM-1, and p-p38 MAPK levels to explore the mechanism of DCBE in treating dry eye. It was shown that the TNF-α, IL-1β, ICAM-1, and p-p38 MAPK levels were significantly weakened in the DCBE groups compared with the positive control in a dose dependent manner, indicating that DCBE can block inflammation signaling transduction to suppress the inflammatory response. However, the exact molecular mechanism remains unclear and requires further investigation in the future.

Conclusion

DCBE showed treatment efficacy in mouse dry eye, which is induced with benzalkonium chloride by blocking the ocular surface squamous metaplasia and alleviating inflammation.

Disclosure of conflict of interest

None.

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References


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