Effects of carvedilol reduce conjunctivitis through changes in inflammation, NGF and VEGF levels in a rat model

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Abstract. Carvedilol is a novel third generation β-blocker that acts as an antagonist of β and α adrenergic receptors, and is able to regulate various cell factors. In addition, it possesses antioxidant activity, is capable of reversing cardiac remodeling effects and has anti-arrhythmic effects. The present study aimed to investigate whether the effects of carvedilol were able to reduce conjunctivitis clinical scores. Initially, 24 Sprague Dawley (SD) rats were randomly divided into three equal groups as follows: Control group, model group and carvedilol group. The model and carvedilol group adult SD rats were injected with lipopolysaccharide (LPS) to induce conjunctivitis. In the carvedilol group, the eight SD rats with LPS-induced conjunctivitis also received 50 mg/kg/day of carvedilol for 4 weeks. Next, the effects carvedilol were assessed utilizing a system of clinical sign scores, and an enzyme-linked immunosorbent assay was used to determine the expression levels of interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNF-α). Finally, nuclear factor-κB (NF-κB), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) were analyzed by western blotting. Carvedilol was observed to significantly reduce clinical sign scores in a dose-dependent manner ($P<0.01$), and reduce IL-1β, IL-6, IL-8 and TNF-α expression levels ($P<0.01$) in the LPS-induced rat model of conjunctivitis. Carvedilol was also able to significantly reduce the protein expression levels of NF-κB, and induce the protein expression levels of NGF and VEGF in the LPS-induced rat model of conjunctivitis ($P<0.01$). In conclusion, the effects of carvedilol may reduce conjunctivitis clinical scores through inflammation, NGF and VEGF in LPS-induced rat models.

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Introduction

Conjunctivitis is the most common allergic eye disease and the most frequently observed ocular surface disease in China (1). According to statistics, ~5% of the global population have sought medical attention due to an allergic eye disease, 50% of which were cases of conjunctivitis (1). Recently, in part as a result of the increased use of eye makeup and the increase of air pollution in certain regions, the incidence of conjunctivitis has increased (2).

Conjunctiva is a transparent layer of mucosal tissue, rich in nerves and blood vessels, that is important for eye protection and lubrication, serving as the protective film of the eye (3). Conjunctivitis can be classified as infectious or non-infectious, with infection taking precedence as the most common cause (4). Pathogenic microorganisms, including bacteria (such as chlamydia, rickettsia), viruses, fungi and parasites (5), are typically transmitted through air, water, physical contact or via tissue adjacent to the affected area by inflammation (including the cornea, eyelid and lacrimal apparatus) (6). Non-infectious conjunctivitis is typically a result of physical stimuli (namely dust mites, smoke, dust and ultraviolet light) or chemical stimuli (including poisonous gas, medical drugs, medical drugs, cosmetics and shampoos) (7).

Although chronic conjunctivitis displays subjective symptoms and involves a characteristically slow disease progression compared with acute conjunctivitis, relapse is common (8). Upon further inspection mild conjunctival congestion edema, papillary hyperplasia, a small amount of white mucous or secretions, such as foam samples may be observed; if not treated promptly, chronic conjunctivitis may develop into carcinomas able to infiltrate the cornea and affect vision (9). Therefore, chronic conjunctivitis requires attention, and further investigation should be performed to identify its causes, eliminate any responsible pathogenic factors, and develop the necessary targeted therapies (10).

Carvedilol is a novel non-selective adrenergic receptor blocker that displays no inherent sympathetic activity. Therefore, the drug bypasses the reflexive excitement of the nervous system often caused by peripheral vascular contraction and increased peripheral resistance. In addition, carvedilol is an oxygen free-radical scavenger that regulates cell factors, such as interleukins and colony stimulating factors, and is involved in a variety of actions (11,12). However, the effect of carvedilol on conjunctivitis has rarely been reported.

Accordingly, the present study aimed to evaluate whether the known effects of carvedilol are able to reduce conjunctivitis clinical scores.

Materials and methods

Animals and modeling. Adult Sprague Dawley (SD) rats (n=24; purchased from the Animal Experimental Center of Zhejiang University, Zhejiang, China) weighing 230-300 g were housed in a 12-h light/dark cycle at a temperature of 22-26˚C, and were provided with food and water *ad libitum*. The study was approved by the ethics committee of Zhejiang University School of Medicine (Huangzhou, China). All SD rats were anesthetized with 75 mg/kg ketamine (injectable IMALGENE 1000; Merial, Lyon, France) and 10 mg/kg xylazine (Rompun 2%; Beyotime Institute of Biotechnology, Haimen, China) by intraperitoneal injection (i.p.). Next, each eye of the anesthetized rats was subconjunctivally injected with 30 μl lipopolysaccharide (LPS; Sigma-Aldrich Química S.A., Tres Cantos, Spain). Further treatment of the rats with LPS-induced conjunctivitis commenced after 24 h.

Study groups. SD rats were randomly divided into three equal groups: i) Control group, consisting of eight SD rats treated with 1 ml 0.9% NaCl (i.p.) for 4 weeks (i.p.); ii) a model group, consisting of eight LPS-induced conjunctivitis rats administered with 1 ml 0.9% NaCl (i.p.) for 4 weeks; and iii) the carvedilol treatment group, consisting of eight LPS-induced conjunctivitis rats treated with 50 mg/kg/day carvedilol (i.p.; Sigma-Aldrich, St. Louis, MO, USA) for 4 weeks.

Evaluation of clinical sign scores. Prior to the euthanasia of the SD rats, clinical sign scores were graded by three blind independent evaluators, as previously reported (13). The clinical score signs were recorded prior to induction of conjunctivitis, and 1, 2, 3 and 4 weeks after carvedilol administration. Clinical sign scores were obtained by three blind independent observers according to previously described criteria (14).

Evaluation of interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) with enzyme-linked immunosorbent assay (ELISA). After 4 weeks of treatment with carvedilol, rats were sacrificed by cervical dislocation. Then, peripheral blood was collected from the eye sockets of the rats. Blood samples were centrifuged at 3,000 x g for 20 min, and the supernatant was collected and stored at -80˚C for further assessment. IL-1β (ml028611), IL-6 (ml002828), IL-8 (ml027376) and TNF-α (ml002859) expression levels were measured using ELISA, according to the manufacturer's protocol (Shanghai Boya Biotechnology Co., Ltd., Shanghai, China).

Evaluation of nuclear factor-κB (NF-κB), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) expression levels with western blotting. After 4 weeks of treatment with carvedilol, rats were sacrificed by cervical dislocation. Then, conjunctivitis tissue samples were collected and homogenized in modified RIPA buffer (pH 7.4; Beyotime Institute of Biotechnology). Cytosolic protein samples were centrifuged at 12,000 x g for 10 min at 4˚C and the supernatant was collected. Protein concentration was determined using a BCA assay kit (Shanghai Boya Biotechnology Co., Ltd.) and equal quantities of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at room temperature, then transferred by electroblotting to nitrocellulose membranes (Hybond-C Extra; GE Healthcare Life Sciences, Pittsburgh, PA, USA). The nitrocellulose membranes were blocked in blocking buffer (Tris-buffered saline and 0.1% Tween-20; Beijing Biosntech Biotechnology Co., Ltd., Beijing, China) containing 5% skim milk, incubated with polyclonal rabbit anti-NF-κB (1:500; sc-7151), polyclonal rabbit anti-NGF (1:2,000; sc-33602) and polyclonal rabbit anti-VEGF (1:1,000; sc-507) (all purchased from Santa Cruz Biotechnology Inc., Dallas, TX, USA) antibodies, washed and then incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000; sc-45101; Santa Cruz Biotechnology Inc.). The resultant bands were visualized by chemiluminescent detection (ECL Western Blotting Detection Reagent; GE Healthcare, Chalfont, UK) and analyzed using a computer imaging system (GDS-8000 Image Acquisition and Analysis System; UVP, LLC, Upland, CA, USA). The reactions were repeated 3 times.

Statistical analysis. All results are expressed as the mean ± standard deviation, and analysis was performed using two-way analysis of variance on the computer program SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA). A value of $P<0.05$ was considered to indicate a statistically significant difference.

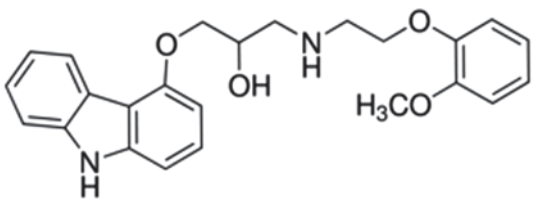

Figure 1. Chemical structure of carvedilol.

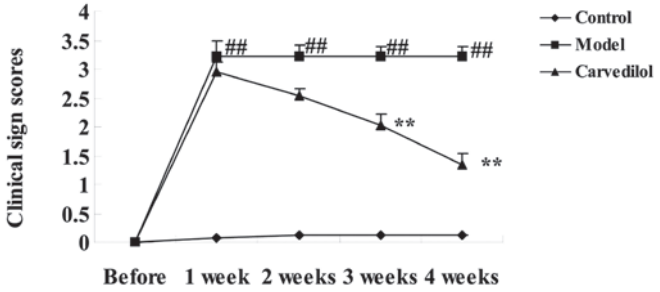

Figure 2. Effects of carvedilol on clinical sign scores in rat models of conjunctivitis. ##P<0.01 vs. control group; **P<0.01 vs. model group. Data are presented as mean ± standard deviation.

Results

Effects of carvedilol on clinical sign scores in conjunctivitis rat models. The chemical structure of carvedilol is displayed in Fig. 1. The effects of carvedilol on clinical sign scores was examined. LPS significantly increased clinical sign scores in the conjunctivitis rat model group compared with the control group ($P<0.01$). By contrast, carvedilol significantly blocked

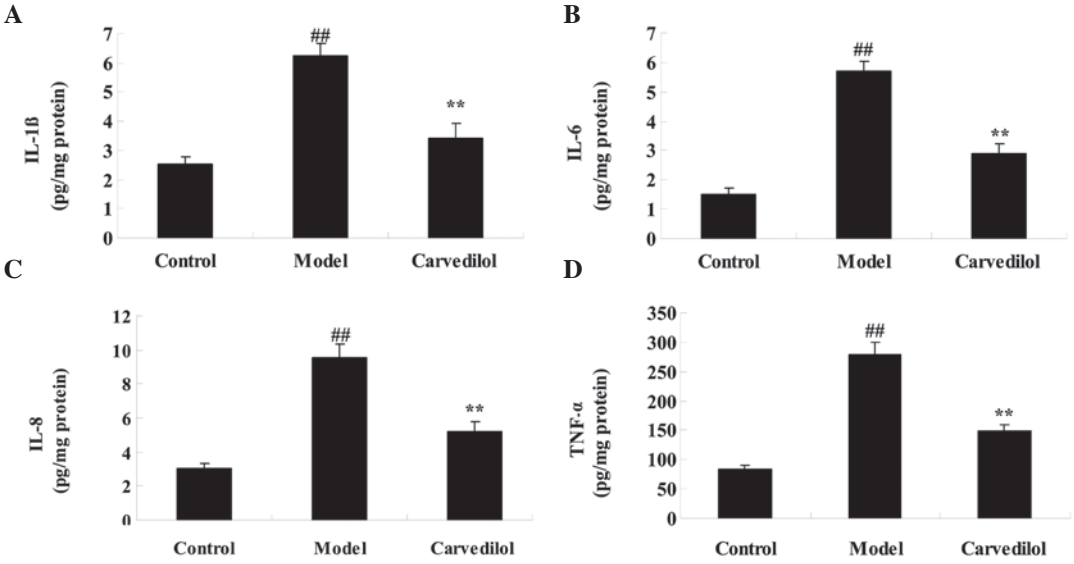

Figure 3. Effects of carvedilol on (A) IL-1β (B) IL-6 (C) IL-8 and (D) TNF-α in a rat model of conjunctivitis. ##P<0.01 vs. control group; **P<0.01 vs. model group. Data are presented as mean ± standard deviation. IL, interleukin; TNF-α, tumor necrosis factor-α.

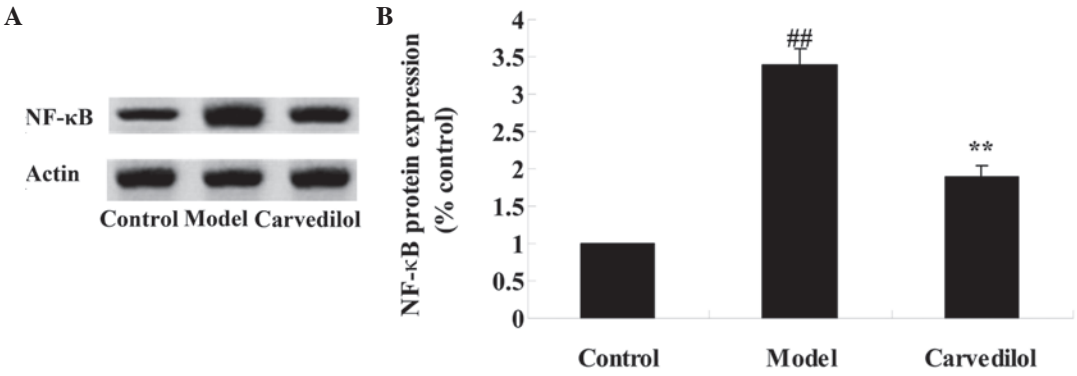

Figure 4. Effects of carvedilol on NF-κB protein expression levels in a rat model of conjunctivitis using (A) western blot analysis and (B) statistical analysis. ##P<0.01 vs. control group; **P<0.01 vs. model group. Data are presented as mean ± standard deviation. NF-κB, nuclear factor-κB.

the increase of clinical sign scores compared with the model group in a time-dependent manner (P<0.01; Fig. 2).

Effects of carvedilol on IL-1β, IL-6, IL-8 and TNF-α expression levels in conjunctivitis rat models. To evaluate the effects of carvedilol on inflammation, IL-1β, IL-6, IL-8 and TNF-α expression levels were analyzed by ELISA. As displayed in Fig. 3, IL-1β (Fig. 3A), IL-6 (Fig. 3B), IL-8 (Fig. 3C) and TNF-α (Fig. 3D) levels were significantly increased in the conjunctivitis rat model compared with the control group (P<0.01). Treatment with carvedilol significantly reduced the augmented expression of all these inflammatory factors compared with the conjunctivitis model group (P<0.01; Fig. 3).

Effects of carvedilol on NF-κB expression in conjunctivitis rat models. To determine the effects of carvedilol on NF-κB protein expression levels in conjunctivitis rat models, western blotting (Fig. 4A) and statistical analysis of the blots (Fig. 4B) was performed. The results revealed significantly increased protein expression levels of NF-κB in the conjunctivitis rat model compared with the control group (P<0.01). However, treatment with carvedilol significantly suppressed NF-κB protein expression levels compared with the model group (P<0.01; Fig. 4).

Effects of carvedilol on NGF expression levels in conjunctivitis rat models. To estimate the effects of carvedilol on NGF protein expression levels in conjunctivitis rat models, western blotting (Fig. 5A) and statistical analysis of the blots (Fig. 5B) was performed. This revealed that conjunctivitis in rat models resulted in inhibition of protein expression levels of NGF in the model group compared with the control group. The administration of carvedilol significantly increased the protein expression levels of NGF in the carvedilol-treated group compared with the model group (Fig. 5).

Effects of carvedilol on VEGF in conjunctivitis rat models. To evaluate the effects of carvedilol on VEGF protein expression levels in rats with LPS-induced conjunctivitis, western blotting (Fig. 6A) and statistical analysis of the blots (Fig. 6B) was performed. The model group displayed significantly reduced expression levels of VEGF compared with the control group (P<0.01). The VEGF protein expression levels

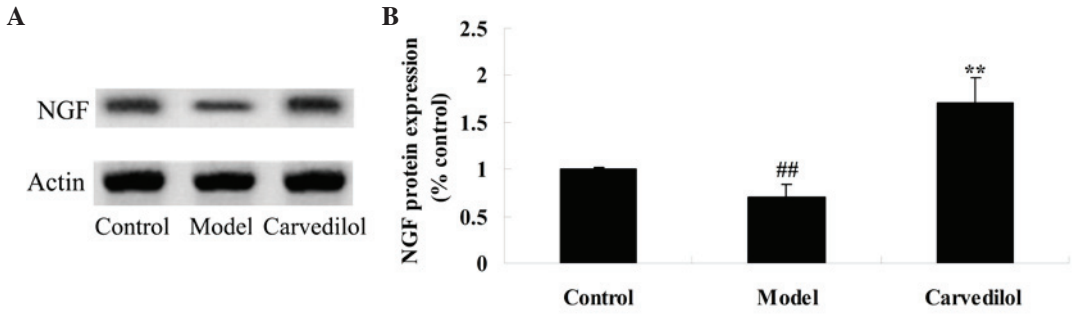

Figure 5. Effects of carvedilol on NGF protein expression levels in a rat model of conjunctivitis using (A) western blotting assays and (B) statistical analysis. ##P<0.01 vs. control group; **P<0.01 vs. model group. Data are presented as mean ± standard deviation. NGF, nerve growth factor.

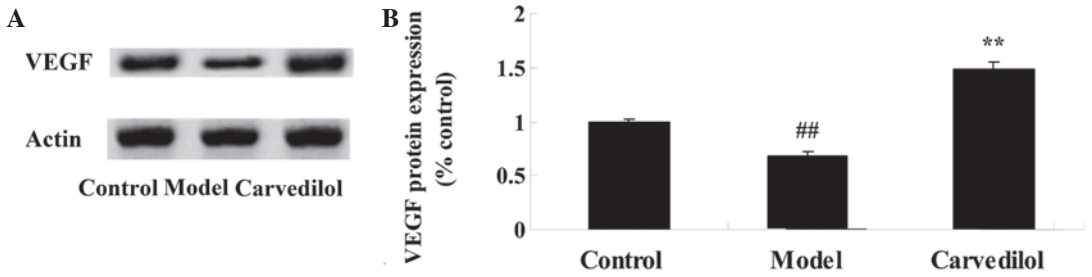

Figure 6. Effects of carvedilol on VEGF protein expression levels in a rat model of conjunctivitis using (A) western blotting assays and (B) statistical analysis. ##P<0.01 vs. control group; **P<0.01 vs. model group. Data are presented as mean ± standard deviation. VEGF, vascular endothelial growth factor.

were significantly reduced in the conjunctivitis model group compared with the carvedilol group (P<0.01; Fig. 6).

Discussion

Conjunctivitis is a frequently observed allergic eye disease. In total, ~5% of the population have sought medical advice as a result of an allergic eye disease, of which >50% were cases of conjunctivitis (15). In recent years, due to increases in factors such as air pollution and the use of contact lenses, the incidence of conjunctivitis has risen (16). Patients typically present with itchiness of the eye, photophobia, lacrimation and a foreign body or burning sensation. Although the majority of conjunctivitis cases rarely result in severe complications, repeated incidence of the disease are common, and cause severe eye discomfort and itching, which may affect the patient's quality of life and work efficiency by reducing their ability to concentrate (17). To the best of our knowledge, the present study revealed for the first time that carvedilol significantly reduces augmented clinical sign scores in a rat model of conjunctivitis.

A recent study revealed that conjunctival infection can cause systemic inflammatory responses involving the induction of inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α (18). It was previously identified that IL-1β is an important inflammatory cytokine, and participates in inflammatory responses to injury and autoimmune diseases. IL-1β, IL-6, IL-8, TNF-α and neutrophils are also involved in an autocrine loop of inflammatory mediators (19). Patients commonly develop with acute conjunctivitis as a result of bacterial or viral infections, which trigger inflammation, and cause mononuclear cells to produce high levels of IL-1β, IL-6, IL-8 and TNF-α. This results in elevated levels of the aforementioned inflammatory cytokines in the blood plasma. Furthermore, cell response function is reduced in acute conjunctivitis, resulting in the disruption of the cellular immune network. In particular, disorders may occur in the percentage of T and B lymphocytes, resulting in increased plasma expression levels of inflammatory cytokines (20). In the present study, elevated IL-1β, IL-6, IL-8, TNF-α and NF-κB expression levels were revealed to be significantly suppressed following treatment with carvedilol. Similarly, de Araújo Júnior *et al* demonstrated that carvedilol decreased IL-1β and TNF-α expression levels in a rat model of periodontitis (21). Additionally, Arab and El-Sawalhi reported that carvedilol alleviated adjuvant-induced arthritis inflammatory mediators (22). Thus, the effect of carvedilol on conjunctivitis may involve the suppression of inflammatory mediators in rats.

Conjunctivitis is a complex inflammatory response involving a variety of factors. Several non-immune factors, including nerve- and endocrine-related factors, may affect the pathological changes and clinical manifestations of conjunctivitis (23). In the serum of patients with conjunctivitis, NGF expression increases significantly; this appears to be associated with the infiltration of mast cells in the palpebral conjunctiva (24). Patients with conjunctivitis also displayed increased levels of neuropeptide, which are synthesized and released by various cell factors involved in NGF regulation and control. Thus, the increased expression levels of NGF appear to result in increased neuropeptide expression in patients with conjunctivitis (25). The phenomenon of increased NGF expression levels is not exclusive to conjunctivitis but also exists in other systemic allergic diseases (26). In the present study, it was revealed that carvedilol significantly increases the suppression of NGF protein expression levels in rat models

of conjunctivitis. Shyu *et al* revealed that NGF mRNA and protein expression levels were upregulated by carvedilol in pressure-overloaded rat hearts (27) and a rat model of volume-overload heart failure (28). These results indicate that the effect of carvedilol on inflammatory mediators may serve to upregulate NGF signaling.

It has been revealed that epithelial cells, inflammatory cells (eosinophils, monocytes/macrophages) and conjunctival fibroblasts produce VEGF following stimulation (29). In angiogenesis, VEGF in epithelial cells serves a core role in vascular matrix change (30). Fibroblasts are another source of the VEGF (31). In the present study, it was demonstrated that carvedilol significantly increases VEGF protein expression levels in rat models of conjunctivitis. Similarly, de Boer *et al* previously reported that carvedilol increased VEGF expression levels in patients with chronic heart failure (32). The results indicate that the effect of carvedilol on conjunctivitis may involve the upregulation of VEGF.

In conclusion, carvedilol is able to reduce the symptoms of conjunctivitis in rat models, and its effect may be associated with the reversal of the abnormal regulation of inflammation, as well as increased NGF and VEGF expression levels, in rats with conjunctivitis. Carvedilol may, therefore, be a potential novel therapy for conjunctivitis.

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