

Heparin Treatment for Allergic Conjunctivitis in the Experimental Balb/c Model

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Key Words

Heparin · Allergic conjunctivitis · Balb/c mice

Abstract

Purpose: To compare the inhibitory effects of dexamethasone and heparin on the infiltration of mast cells in the conjunctiva by using a mouse allergic conjunctivitis model.

Methods: 24 Balb/c mice were divided into four groups: allergy group (positive control), dexamethasone group, heparin group, and negative control group, as groups 1, 2, 3, and 4, respectively. Each group comprised 6 mice, and experimental allergic conjunctivitis was developed in groups 1, 2, and 3. The mice in group 2 were treated with topical 0.1% dexamethasone eye ointment, and the mice in group 3 were treated topically with 5,000 IU/ml standard heparin. Both dexamethasone and heparin were instilled once a day for 4 days. Hanks' balanced salt solution was dropped into both eyes of the mice in group 4 instead of dexamethasone or heparin. Eyeballs and eyelids were removed from the mice in all groups while one eye of each animal was used for histopathological, the other for molecular biological examination. **Results:** Mast cells, infiltrating the subconjunctival tissue, were significantly lower in group 2 ($p < 0.0001$), group 3 ($p < 0.0001$) and group 4 ($p < 0.0001$) when compared to

group 1. **Conclusions:** Topical heparin could be a viable option in treating IgE-induced allergic eye disease since it is found to be as effective as topical dexamethasone in experimental acute allergic eye disease.

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Introduction

The endogenous glycosaminoglycan heparin is present in high concentrations in preformed cytoplasmic mast cell granules and released together with histamine and able to interact with a variety of molecules in vivo, because of its flexible structure and high anionic charge [1]. Distinct from the well-known anticoagulant properties, heparin has multiple nonanticoagulant properties such as anticomplement, anti-inflammatory, antiatherosclerotic, immunoregulatory and anti-allergic activities [1–7]. Histamine release is inhibited by incubation of basophils with heparin [8]. Heparin inhibits allergen-induced eosinophil recruitment in the guinea pig lung via a mechanism unrelated to its anticoagulant activity [9]. Inhaled heparin and low-molecular-weight heparin inhibit methacholine-induced bronchoconstriction [10] and exercise-induced asthma [11].

Dolowitz and Dougherty [12] reported the first subjective improvement in asthma symptoms with using intravenous heparin in 1960. Bardana et al. [13] performed the first trial of inhaled heparin observing subjective improvement. Ahmed et al. [14] suggested that heparin inhibits IgE and inositol-1,4,5-triphosphate (IP₃)-induced mast cell histamine release. Ahmed et al. [15] have also demonstrated that pretreatment with 1,000 U/kg heparin attenuated the increase in the lung resistance of previously sensitized sheep by 91% on allergen reexposure. Moreover, Bendstrup and Jensen [16] reported 2 cases of corticosteroid-resistant asthma patients who responded to 100,000 units inhaled heparin during asthma exacerbations. Besides asthma treatment, heparin has been used for the treatment of allergic contact dermatitis [17], but there are only preliminary reports on the use of heparin in ocular allergy [18]. Corticosteroid treatment has to be given to some patients for whom antihistaminics are not enough to control their ocular allergy. However, corticosteroids have serious side effects, such as steroid-induced glaucoma, causing cataracts and potential corneal infections, and so should not be used routinely in these conditions. Also it is known that in spite of the fact that topical steroids are effective in reducing the influx of inflammatory cells, they have little effect on mast cell mediator secretion. Administration of some immunosuppressive agents is under study [19–23] but the therapeutic mechanism of these drugs for reducing the clinical manifestations of allergic eye diseases still remains uncertain.

In this study, we compared the inhibitory effects of dexamethasone and heparin on the infiltration of mast cells in the conjunctiva using a mouse allergic conjunctivitis model.

Materials and Methods

Mouse Model for Experimental Allergic Conjunctivitis and the Drug Instillation

Method

Balb/c mice (24 males), aged 8–12 weeks, were obtained from Adnan Menderes University Pharmacology Department, Aydın. The protocol of the study was approved by the local ethical committee, and the Animal Experiments Ethics Review Committee of the Adnan Menderes University. All mice were treated in accordance with the Association for Research in Vision and Ophthalmology resolution regarding animals used in research. The mice were divided into the following four groups: the allergy group (positive control), dexamethasone group, heparin group, and the negative control group, as groups 1, 2, 3, and 4, respectively. Each group comprised 6 mice.

Experimental allergic conjunctivitis in mice was developed by the following method (table 1). On the 1st and 8th days, a 0.2-ml

Table 1. Sensitization, antigen challenge and treatment protocols of the four groups

	SS + OVA IP	SS IP	HBSS + OVA T	HBSS T	Dexame- thasone T	Heparin T
Group 1 (n = 6)	+	–	+	–	–	–
Group 2 (n = 6)	+	–	+	–	+	–
Group 3 (n = 6)	+	–	+	–	–	+
Group 4 (n = 6)	–	+	–	+	–	–

SS = Saline solution; OVA = ovalbumin; IP = intraperitoneal; HBSS = Hanks' balanced salt solution; T = topical.

mixed solution, adjusted to a concentration of 5 mg/ml of ovalbumin and 15 mg/ml of aluminum hydroxide, was administered intraperitoneally to the mice in groups 1, 2, and 3 for sensitization as described below. On the 1st and 8th days, a 0.2-ml saline solution was instilled intraperitoneally to the mice in group 4.

To induce experimental allergic conjunctivitis, an antigen challenge was made on days 15 and 18, using an ovalbumin solution (5 mg/ml) instilled into both eyes of the mice in groups 1, 2, and 3.

From day 15 to 18, the mice in group 2 were treated with topical 0.1% dexamethasone eye ointment (Maxidex, Alcon-Couvreur, Belgium), and the mice in group 3 were treated topically with 5,000 IU/ml standard heparin (Nevparin, Mustafa Nevzat Pharmaceuticals, Turkey). Both dexamethasone and heparin were instilled once a day for 4 days. The mice in group 4 received Hanks' balanced salt solution into both eyes instead of dexamethasone or heparin.

Twelve hours after the last instillation of ovalbumin solution, eyeballs and eyelids were removed from the mice of all groups, following euthanasia by thiopentone sodium (pentothal sodium; Abbott Laboratories Inc.). One eye of each animal was used for histopathological, the other for molecular biological examination.

Histopathological Examination

All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin according to the routine tissue processing method of the laboratory. In addition to hematoxylin-eosin (HE), and toluidine blue (TB), 4-μm-thick stained sections were placed on ready-to-use adhesive glass slides (MicroSlides Snowcoat X-tra; Surgipath, Richmond, Ill., USA) for mast cell tryptase.

Immunohistochemical examination was carried out with a commercial detection kit using the labeled streptavidin-biotin amplification method and 3,3'-diaminobenzidine as chromogen (Histostain-Plus kits, cat. No. 85-9643; Zymed Laboratories, South San Francisco, Calif., USA). Sections were tested by using mouse anti-human monoclonal antibodies against mast cell tryptase. Sections were deparaffinated in xylene and dehydrated through a graded series of ethanol solutions, followed by microwave antigen retrieval for 20 min in 0.01 M sodium citrate buffer. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide for 15 min. After washing in Tris-buffered saline (pH 7.6), serum blocking was performed with ready-to-use solution of the kit for 10 min. The sections were incubated for 1 h with the primary antibody, washed in Tris-buffered saline, incubated in an appropriate biotinylated secondary antibody for 10 min and then washed

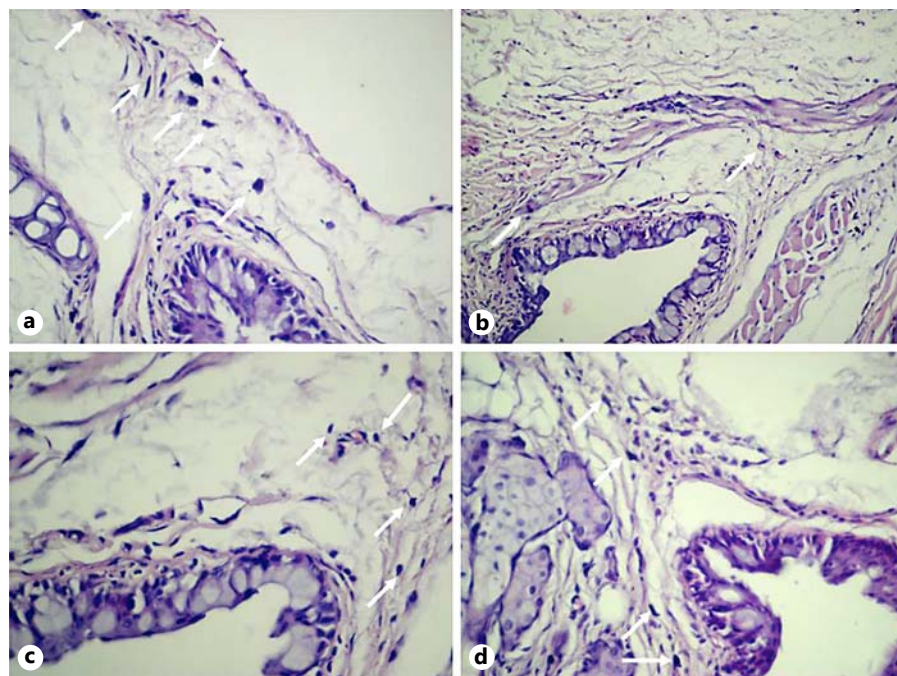


Fig. 1. Histopathological sections of conjunctiva showing mast cells (arrows) in the subconjunctival tissue. HE. $\times 40$. **a** Group 1 (positive control). **b** Group 4 (negative control). **c** Group 2 (dexamethasone). **d** Group 3 (heparin).

again in Tris-buffered saline. Binding was visualized by incubating the sections with 3,3'-diaminobenzidine and counterstaining lightly with hematoxylin. Finally, the sections were dehydrated and mounted.

HE, TB, and immunohistochemically stained samples were all examined through a light microscope (Olympus BX50, Olympus Optical Co. Ltd., Tokyo, Japan). Mast cells, eosinophils, lymphocytes, and vascular intensity of the subconjunctival tissue were examined under a power of $\times 40$ from 50 separate areas by using a simple micrometric 10×10 grid disk inserted into the eyepiece of the microscope.

Statistical Analysis

Student's *t* test was performed to compare the cell densities in different groups. A *p* value less than 0.05 was considered statistically significant.

Results

Mean mast cell density in cells per square millimeter, infiltrating the subconjunctival tissue, was significantly less in group 2 (dexamethasone; mean $\pm$ SD 8.17 ± 2.69 , $p < 0.0001$), group 3 (heparin; 7.08 ± 2.43 , $p < 0.0001$) and group 4 (negative control; 5.58 ± 3.12 , $p < 0.0001$) when compared to group 1 (23.17 ± 7.46 ; fig. 1–4). There was no increase in eosinophil and lymphocyte counts as well as vascular intensity in the subconjunctival tissue in any group.

The experiment was repeated a second time using 6 mice for each group, and basically similar results were obtained.

Discussion

Both acute and chronic allergic conjunctivitis is prevalent worldwide [24]. The usual medical approach involves antihistaminic topical medications as a first-line therapy and topical corticosteroid medication is added if necessary. However, especially chronic use of topical corticosteroids may lead to the development of well-known side effects including cataract and glaucoma. Side effects of heparin are hypersensitivity reactions such as urticaria, conjunctivitis, rhinitis, asthma, angioedema and anaphylactic shock, priapism, bleeding, rash, thrombocytopenia, alopecia, anaphylaxis, rigor or slight hematuria. We have not seen any of these side effects in mice. There is no study investigating heparin treatment for allergic conjunctivitis. However, we have not studied dose response experiments yet; further studies are needed to clarify the side effects related with dose responses.

Conjunctival biopsy in allergic eye disease is characterized by numerous metachromatic staining mast cells [25]. Tissue mast cells play a major role in type I hypersensitivity reactions. Using the presence of tryptase, a unique

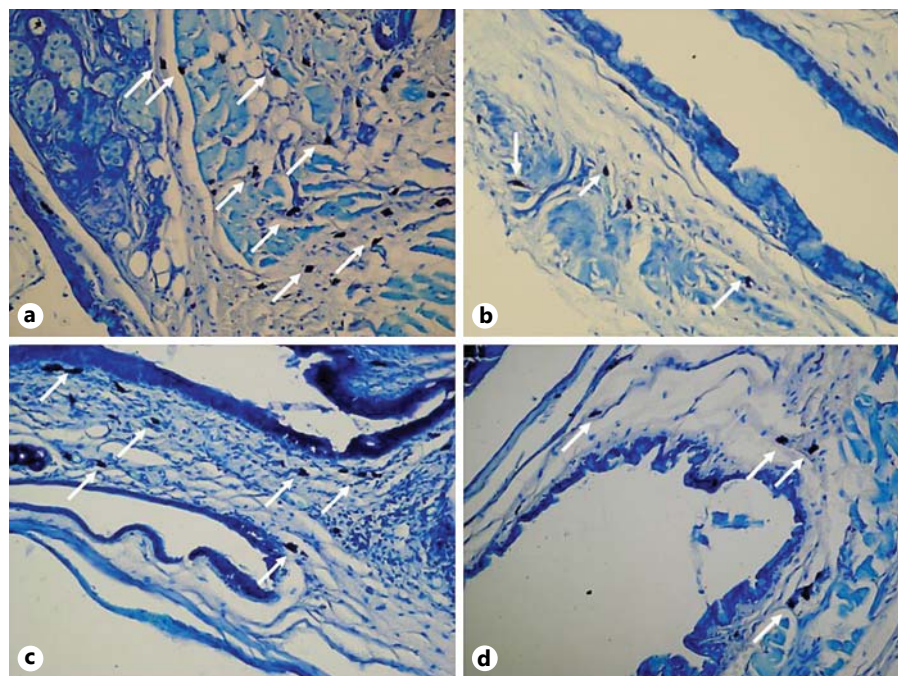


Fig. 2. Histopathological sections of conjunctiva showing mast cells (arrows) in the subconjunctival tissue. TB, $\times 40$. **a** Group 1 (positive control). **b** Group 4 (negative control). **c** Group 2 (dexamethasone). **d** Group 3 (heparin).

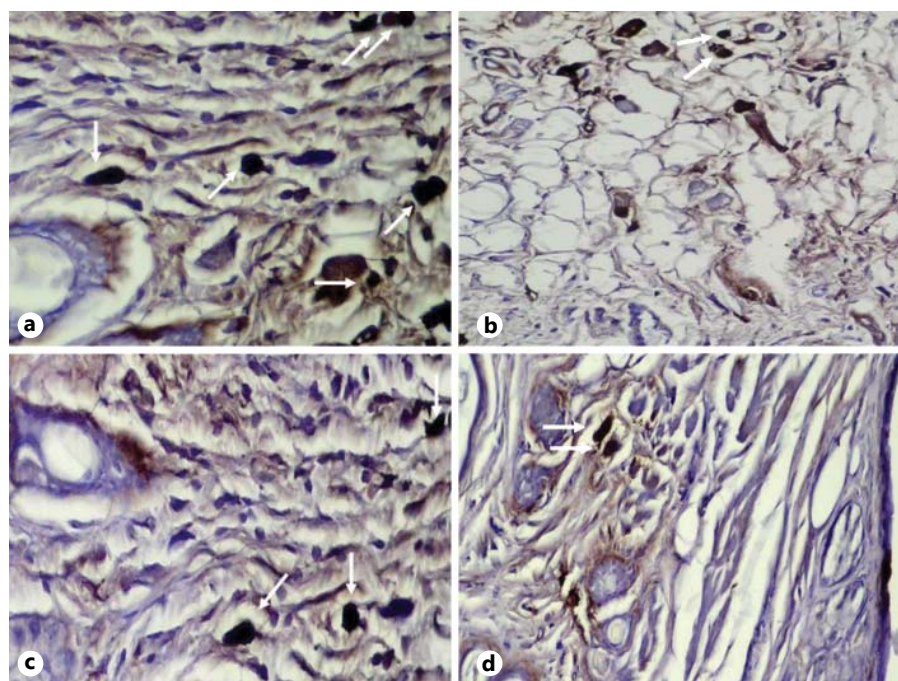


Fig. 3. Immunohistochemical staining of conjunctival tissues for mast cell tryptase (arrows). $\times 40$. **a** Group 1 (positive control). **b** Group 4 (negative control). **c** Group 2 (dexamethasone). **d** Group 3 (heparin).

neutral protease, as an immunohistochemical marker, mast cell numbers have been found to have increased in all types of allergic diseases [26–28], suggesting the contribution of IgE-mediated mast cell activation to all of these conditions. The mast cell is central to the mecha-

nism of allergic eye disease, triggering the ‘acute reaction’, and continued activation of this reaction is followed by a late-phase response. In addition, mast cells are an important source of multifunctional cytokines in allergic eye disease, which also enables them to have a central role in

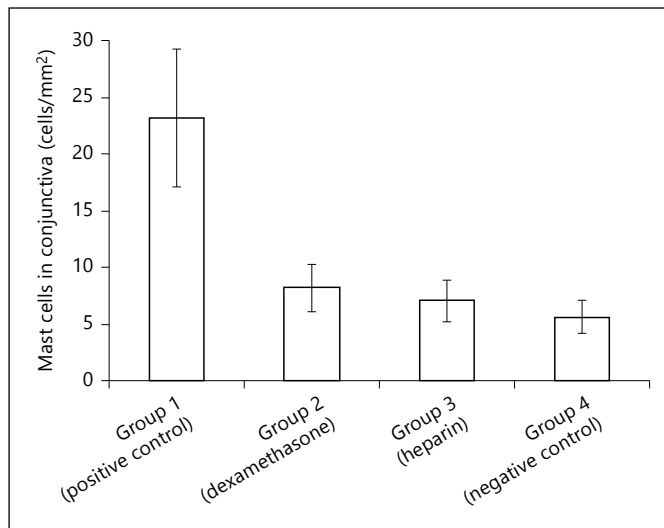


Fig. 4. Effects of dexamethasone and heparin treatment on mast cell infiltration in the conjunctival tissues. The difference between group 1 (positive control) and all the other groups was statistically significant (Student's *t* test, *p* < 0.0001). Bars represent standard deviations.

the disease mechanism. Their degranulation leads to not only the immediate symptoms of seasonal allergic conjunctivitis, but also to a rapid upregulation of conjunctival inflammation in all forms of ocular allergy through the IgE-dependent release of stored cytokines [29].

Other conjunctival cells also contribute to the disease process, one of which is the eosinophil. Shoji et al. [30] have reported that topical dexamethasone and FK506 treatments were efficacious in suppressing both eosinophil and lymphocyte infiltration into subconjunctival tissue in the mouse experimental allergic conjunctivitis model. Acute allergic conjunctival disease is characterized by infiltration of mast cells, as in our model, but not eosinophils or lymphocytes [31–34]. However, while the presence of mast cells is strongly indicative of allergic eye disease, its absence in conjunctival scrape cytology does not preclude the diagnosis [35]. In normal conjunctiva, eosinophils are absent in the epithelium of nonatopic subjects [35], but their numbers in conjunctival epithelium, subepithelium, and tears are increased to a great extent in chronic allergic eye disease such as vernal keratoconjunctivitis [36]. Platelet-activating factor and eosinophilic chemotactic factor of anaphylaxis, both of which are produced by mast cells, are chemotactic for eosinophils that join the process later than mast cells. Thus, eosinophils are not expected to be seen in the acute phase but rather in the chronic phase of allergic conjunctivitis.

Correspondingly, in our study, there was no increase in eosinophils, lymphocytes, or vascularization in the subconjunctival tissue. Other forms of inflammation were not seen in the histopathological examination.

The pro-inflammatory effects of histamine on the conjunctiva, which induces both the production of interleukin (IL)-1, IL-6, and IL-8 and the expression of intercellular adhesion molecule-1, cannot be maintained by inhibiting histamine release [37]. Although the topical steroids have little effect on mast cell secretion, heparin stops the cascade from the beginning by inhibiting specifically histamine release from the mast cells. Heparin may serve as an endogenous mediator that provides negative feedback on the release of other pro-inflammatory mediators from the mast cell, besides histamine. Ahmed et al. [38] suggested that the protective effect of heparin on exercise-induced asthma may be related to inhibition of IP₃-dependent stimulus secretion coupling in mast cells and thus heparin may form a basis of therapeutic approaches. It has been proposed that the antiallergic activity of unfractionated heparin may be mediated by inhibition of IP₃-dependent release [14, 39]. Low-molecular-weight heparins have also been shown to inhibit IP₃-induced Ca²⁺ release [40] and to attenuate IgE-induced mast cell degranulation [41]. It has been observed that inhibition of IP₃ binding to its receptors by heparin is molecular weight dependent, and the inhibitory activity decreased as the size of the heparin chain was reduced below 18 monosaccharide units [32]. In our study, we used high-molecular-weight heparin and found it to be effective in preventing the population of mast cells in subconjunctival tissue in experimental acute allergic eye disease.

Heparin is used in ophthalmology to inhibit fibrin reaction and to prevent endophthalmitis. Also some intraocular lenses are coated with different molecular weights of heparins. Different molecular weight heparins act differently from each other by means of penetrating the anterior chamber. It is possible to calculate for all heparin types. For instance, Mannens et al. [42] used doses from 5 IU/ml and Gills [43] from 1.6 IU/ml.

The therapeutic effect of heparin is based on many studies and therapeutic trials [38, 44–51]. The concept of our thesis has been seen in other allergic responses [38, 44–51]. Anti-inflammatory and antiallergic properties of heparin have been demonstrated in several *in vitro* and *in vivo* studies [5–7, 15]. However, no research has been undertaken on the treatment of allergic conjunctivitis.

Heparin is a highly sulfated glycosaminoglycan and present in mast cells at high concentrations [44]. Heparin is also a member of the linear anionic polyelectrolytes

which have individual highly negatively charged chains [47]. These compounds show a wide spectrum of specific reactions with biologically active proteins [47]. Heparin can restore the electronegativity of the vessels by various compounds, including histamine, angiotensin and bacterial endotoxin, and has antioxidant properties [47]. Heparin has different roles in many clinical uses and in physiological and pathological responses [47].

Schultz et al. [44] suggested that the protective activity of intranasal heparin seemed to be related to the neutralization of eosinophil cationic protein, as well as to the reduction of eosinophil recruitment. Intranasal heparin significantly reduced the symptom score 10 min after antigen challenge in allergic rhinitis [51]. The underlying molecular mechanisms seem to be complex and certainly need further investigation.

Page [50] suggested that heparin serves a defensive role in the airways. Pavord et al. [45] reported that this defensive role does not extend to inhibition of neurally induced bronchoconstriction.

Ahmed et al. [46] studied the pharmacodynamics of the antiallergic action of heparin. They found that aerosol and intravenous heparin attenuated the antigen effects [46]. They also reported an inhibitory effect of inhaled heparin on allergen-induced bronchoconstriction in *Ascaris suum*-sensitive sheep in less than 20 min after inhalation. Further, they suggested that heparin has a number

of anti-inflammatory effects including inhibition of mast cell mediator release. The mechanism of action is unclear, although studies suggest that heparin inhibits mast cell mediator release [46].

Bowler et al. [48] showed that intravenous heparin significantly inhibited the cutaneous response to antigen, and that in subjects with asthma and dust mite allergy, nebulized heparin significantly reduced the bronchospasm induced by inhaled dust mite extract. Another study [49] confirmed the benefit of inhaled heparin in patients with atopic asthma.

There is no heparin study for allergic eye disease. This is the only study in the literature where you could say that 'heparin is effective for allergic eye disease like steroids'. Our animal model is a similar model with other animals [30] in the literature; other forms of inflammation were not seen in histopathological examination. These types of studies need to be conducted with different animals before human trials. Our paper is a fundamental study which will shed light on future studies.

The results of our study imply that topical heparin could be a viable option in IgE-induced allergic eye disease that is found to be as effective as topical dexamethasone in experimental acute allergic eye disease. Further clinical studies are needed to determine the safety and effectiveness of topical heparin and to clarify the dose responses and side effects.

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