Protective Effect of d-β-Hydroxybutyrate on Corneal Epithelia in Dry Eye Conditions through Suppression of Apoptosis

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Purpose. To investigate the effect of d-β-hydroxybutyrate (HBA) on ocular surface epithelial disorders induced by tear fluid deficiency, the potency of HBA and serum, the efficacy of which has been well documented in clinical application, were compared.

Methods. Rat corneal epithelial erosion was induced by exposure of rat eyes to continuous low-humidity airflow, which accelerated the tear evaporation. During desiccation, one eye of each rat was treated with HBA (20, 40, or 80 mM) or rat serum (5%, 20%, or 100%), and in the other eye a drop of phosphate-buffered saline (PBS) was instilled as the control. Histopathologic examination and quantification of the epithelial defect area were performed. The apoptosis in the epithelia was determined by chromatin condensation using the Hoechst 33342 fluorescein probe.

Results. In PBS-treated eyes, thinning in the cell layer was seen on the periphery of the initial wound after 6 hours, and it progressed to defects after 12 hours. In the 80-mM HBA and 20% serum applications, the pathologic change in the epithelia was moderate, and the structure was maintained in an almost normal state in the 100% serum application. Significant decreases in the defect areas were observed in the 5%, 20%, and 100% serum and 40- and 80-mM HBA treatment groups compared with the PBS-treated eyes (n = 12). A significant suppression of chromatin condensation was observed with HBA and serum treatment.

Conclusions. These results suggest the potential clinical application of HBA for ocular surface epithelial disorders to maintain epithelial cell viability in patients with dry eye. (Invest Ophtalmol Vis Sci. 2003;44:4682–4688) DOI:10.1167/ iovs.03-0198

To date, a variety of clinical approaches have been used to cure abnormalities in tear fluid status in dry eye conditions. For improvement of the decreased volume and instability of aqueous fluid on the ocular surface, frequent application of preservative-free artificial tears or punctal occlusion has been performed in conventional management. In addition, tear replacement therapy with autologous serum has been attempted to correct the insufficient composition of tear fluid along with aqueous fluid, on the basis that the growth factor, cytokine, vitamin, and nutrition content in serum resembles aqueous tears.

Fox et al.² found that the use of a topical application of diluted autologous serum improved symptoms in patients with keratoconjunctivitis sicca and reduced the resistance to therapy with commercially available artificial tears. Autologous serum application has been reported to be effective in the treatment of severe dry eye states associated with ocular pemphigoid, Stevens-Johnson syndrome, and chronic graft-versus-host disease.³–⁵ and persistent epithelial defect.⁶ Recently, investigators in a placebo-controlled study conducted in patients with bilateral severe dry eye reported a trend toward an improvement in symptoms, including cellular changes.⁷ These successful clinical results showed practically that tear replacement therapy using autologous serum is an effective clinical management for dry eye and suggest that the key concept in effective ophthalmic formulations is related to providing a proper environment for the ocular surface cells.¹,⁸ HBA is produced primarily in the liver by the degradation of a long fatty acid and exists abundantly in the plasma and peripheral tissues. The levels in human plasma and tissues are maintained below 0.1 mM in the normal state.⁹ The role of HBA as an oxidative fuel and lipogenic precursor has been well recognized for some time.¹⁰ Because HBA has good penetration and rapidly diffuses in the peripheral tissues, the therapeutic benefits of exogenously applied HBA have been documented under stressful conditions, such as hemorrhagic shock,¹¹,¹² extensive burns,¹³ and cerebral hypoxia, anoxia, and ischemia.¹⁴ In these states, HBA ameliorates tissue damage, protein catabolism, and metabolic dysfunction. Furthermore, HBA has been shown to decrease cell death in human neuronal cell cultures in Alzheimer’s and Parkinson’s disease models.¹⁵ This evidence shows the possible therapeutic efficacy of HBA in a variety of stress-induced conditions.¹⁶ However, the effects of HBA on ocular surface epithelial disorders have not been investigated. In the present study, we used a rat model of dry eye to determine whether topically applied HBA could heal corneal epithelial erosion caused by ocular surface desiccation. In addition, to assess the therapeutic benefit of HBA in ophthalmic formulations, we compared the potency of HBA with serum, the efficacy of which has been well documented in clinical application. We found that HBA, as well as serum, ameliorates the appearance of corneal epithelial erosion through suppression of apoptosis.

Materials and Methods

Animals

Male 8-week-old Sprague-Dawley rats (n = 6–12 in each experiment) were purchased from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo, Japan). They were quarantined and acclimatized before the experiments for a 1-week period under standard conditions: room temperature 23 ± 2°C, relative humidity 60% ± 10%, an alternating

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Submitted for publication February 26, 2003; revised June 2, 2003; accepted June 14, 2003.

Disclosure: S. Nakamura, Ophtecs (E); M. Shibuya, Ophtecs (E); Y. Saito, Ophtecs (E); H. Nakashima, Ophtecs (E); F. Saito, Ophtecs (E); A. Higuchi, None; K. Tsubota, None

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12-hour light–dark cycle (8 AM to 8 PM), with water and food available ad libitum. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Ophthalmic Solutions**

Sodium HBA (Fig. 1) was synthesized by Ophtecs Corporation, Ltd. (Hyogo, Japan), and was found to be more than 99% pure when tested by HPLC. Rat serum was the pooled serum, collected in a sterile microtube, and washed twice with PBS. The cells were incubated in a mixture of ketamine and xylazine and the eyeballs removed and maintained for 12 hours.

**Desiccation Procedure.** Just after scraping, the rats were placed in a desiccation room, with room temperature of 23 ± 2°C, relative humidity of 28% ± 2%, and constant air flow (2–4 m/sec), and maintained for 12 hours.

**Topical Eye Drop Application.** During desiccation, one eye of each rat was treated with HBA or serum eye drops, and the other eye was given a drop of PBS as the control. Ten microliters of eye drops was administered every hour for 12 hours.

**Quantitative Determination of Corneal Epithelial Erosion.** The damaged areas were photographed immediately after and 2, 6, and 12 hours after scraping, by applying a fluorescein solution under cobalt blue light. The stained area (epithelial erosion) was digitized with an optical scanner and quantified with image-analysis software (NIH Image, ver. 1.55; available by ftp at zippy.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**Histopathologic Study.** The rats were killed with an overdose of a mixture of ketamine and xylazine and the eyeballs removed and fixed in Davidson’s solution (37.5% ethanol, 12.5% acetic acid, and 25% formaldehyde [57% solution]). The corneal specimens were embedded in paraffin, cross-sectioned, and stained with hematoxylin and eosin.

**Chromatin Condensation Evaluation in Corneal Epithelium.** The level of apoptosis in the corneal epithelia was quantified by modification of a previously described method. Whole corneal epithelium was scraped with an ophthalmic surgical blade, placed in a microtube, and washed twice with PBS. The cells were incubated in the dark with 10 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) in PBS for 30 minutes (500 μl/tube). After maintaining the rats in a desiccated condition for at least 5 days, we observed corneal epithelial disorder, superficial punctate keratopathy, and erosion in most of the eyes. However, variations in the frequency of appearance, degree of disorder and time of appearance were noteworthy in this condition (Nakamura S, unpublished data, 2000). Thus, to measure the residue tear fluid volume, we attempted a modified phenol red thread test on the rats’ eyes to measure the residue tear fluid volume. During the ocular surface desiccation procedure, a significant decrease in tear fluid was observed compared with the standard condition (Fig. 2).

**Statistical Analysis**

Analysis of the significance of differences was performed on computer by the paired or unpaired Student’s t-test between two groups (StatView IV; Abacus Concepts, Berkeley, CA). Differences were accepted as significant at P < 0.05.

**Results**

**Tear Fluid Volume Measurement**

First, to confirm that our desiccation condition of exposure to a continuous 28% ± 2% humidity airflow at 2 to 4 m/sec induces a deficiency of tear fluid on the ocular surface, we attempted a modified phenol red thread test on the rats’ eyes to measure the residue tear fluid volume. During the ocular surface desiccation procedure, a significant decrease in tear fluid was observed compared with the standard condition (Fig. 2). These results indicate that tear fluid deficiency was induced by exposure to continuous low-humidity airflow due to acceleration of tear evaporation from the ocular surface. Thus, we used this procedure to induce the ocular surface disorder.

**Induction of Corneal Erosion**

After maintaining the rats in a desiccated condition for at least 5 days, we observed corneal epithelial disorder, superficial punctate keratopathy, and erosion in most of the eyes. However, variations in the frequency of appearance, degree of disorder and time of appearance were noteworthy in this condition (Nakamura S, unpublished data, 2000). Thus, to enable us to assess the effect of pharmacological treatments accurately, we attempted to induce uniform corneal epithelial disorder by scraping a small area of the corneal epithelium as a trigger.

After 2 hours, in both the standard and desiccated conditions, the epithelial defect remained in the same area as at the initial level. In the normal condition, the corneal epithelial defect started to decrease after 6 hours (Fig. 3A) and had almost disappeared within 12 hours (Fig. 3C). During the desiccation of the ocular surface, aggravation of the respective area was clearly observed. Six hours after the desiccation was ended, a lightly fluorescein-stained area appeared surrounding...
the initial epithelial defect (Fig. 3B) and progressed to a distinct, extensively stained spot after 12 hours (Fig. 3D). Quantitative analysis of the fluorescein-stained area showed a significant increase in stained area after 6 and 12 hours of treatment compared with that before treatment (Fig. 3E). In the histopathologic examination, a slight corneal epithelial migration to the wounded area was observed after 6 hours, and the defective area was completely covered with multilayer, regenerated epithelium after 12 hours in the standard condition (Figs. 4A, 4C). However, after 6 hours of desiccation, thinning of the cell layer accompanied by extensive exfoliation of the corneal epithelia was seen on the periphery of the initial wound (Fig. 4B), and then these degenerations progressed to defects after 12 hours (Fig. 4D). These observations suggest that desiccation of the ocular surface initially induces exfoliation of the corneal epithelium and results in erosion when these epithelia have entirely detached.

Apoptosis in Corneal Epithelium

Induction of apoptosis on the ocular surface in dry eye has been documented in the conjunctiva and corneal epithelium, although the mechanism involved is still unclear. To confirm apoptosis in the desiccated corneal epithelium, we used Hoechst 33342 staining to examine the state of chromatin, one of the characteristic features of apoptosis, regardless of the apoptotic pathways involved. In addition, to differentiate apoptosis from necrosis, we used an index of chromatin condensation (Ho; Hoechst 33342 fluorescence/cell viability (NR; membrane integrity detection by neutral red assay), as previously described. We assessed the apoptosis after 6 hours of ocular surface desiccation on the cornea, because thinning of the corneal epithelium, an indication of erosion, was apparently observed. Significant increases in Hoechst 33342 fluorescence (Fig. 5A) and the Ho/NR ratio (Fig. 5B) were observed in the desiccation treatment in contrast to the standard condition treatment. These results indicate that the progression of apoptosis in the corneal epithelium plays a critical role in the degeneration of the corneal epithelium that leads to erosion.

Effect of HBA and Serum on the Occurrence of Corneal Epithelial Erosion

Based on the tear replacement therapy using autologous serum for severe dry eye, we used rat serum as a positive control in...
our rat dry eye model. To assess the effect of HBA and serum on the appearance of corneal epithelial erosion, we selected a time point after 12 hours of desiccation, because the appearance of erosion was identical up to this point. As shown in Figure 6, both HBA and serum dose-dependently decreased the fluorescein-stained areas. Significant decreases in the fluorescein-stained areas were observed in all the serum-treated groups (5%; \( P < 0.05 \), 20%; \( P < 0.01 \), 100%; \( P < 0.005 \), Fig. 6A) compared with the PBS group. In the HBA-treated group, a significant decrease was observed at 40 and 80 mM HBA (40 mM; \( P < 0.05 \), 80 mM; \( P < 0.005 \) versus PBS, Fig. 6B). A maximum effect was observed with 80 mM HBA (65% of PBS) and 100% serum (49% of PBS).

Because corneal epithelial degeneration evidently occurs 6 hours after ocular surface desiccation treatment, we used this time point for histopathologic examination. Representative patterns of histopathologic changes in the corneal epithelium after 6 hours are shown in Figure 7. The changes in the features of the epithelial cell layer were consistent with the quantitative analysis data after 12 hours of treatment. In PBS, thinning in the cell layer accompanied by detachment of the corneal epithelial cell layer was observed surrounding the periphery of the wound (Fig. 7A). The thinning of the cell layer was moderate in the 20% serum (Fig. 7B) and 80 mM HBA (Fig. 7C) applications, and the structure of the epithelial cell layer was maintained in an almost normal state in the 100% serum application (Fig. 7D).

These results indicate that the suppressive effects of HBA and serum on the appearance of corneal epithelial erosion were due to a protective potential against epithelial cell degeneration.

**Effect of HBA and Serum on Corneal Epithelial Apoptosis**

To investigate whether the effects of HBA and serum arises from the suppression of apoptosis, we performed a Hoechst 33342 assay on 80 mM HBA and 100% serum applied to the cornea after 6 hours of desiccation. Consistent with the former results, significant decreases in apoptosis, fluorescence intensity, and Ho/NR ratio were observed when 80 mM HBA and 100% serum were applied to the cornea in contrast to PBS treatment. The effect of 100% serum was more apparent than that of 80 mM HBA (Fig. 8).
discussed, therefore, that a synergic effect between enhanced apoptotic signals and insufficient supplementation of essential factors to the corneal epithelia may have been involved in the pathogenesis of corneal epithelial erosion.}

**DISCUSSION**

In this study, we used a novel rat dry eye model to evaluate the effect of HBA on ocular surface epithelial cell disorder. We demonstrated that HBA, as well as serum, ameliorates the appearance of corneal epithelial erosion due to ocular surface desiccation accompanied by suppression of apoptosis.

In our rat model, we found that a mechanically created partial corneal epithelial defect was aggravated to extensive epithelial erosion during exposure to continuous low-humidity airflow on rat eyes, which accelerated the tear evaporation rate and enhanced ocular surface desiccation. Several animal models have been reported to mimic the clinical features caused by tear fluid deficiency on the ocular surface. Increased keratoconjunctivitis sicca, rose bengal staining on the conjunctiva, and/or increased corneal epithelial permeability have been established in monkeys,24 dogs,25–28 rabbits,29,30 rats,31 and mice.32 However, to our knowledge, tear fluid depletion-induced corneal epithelial erosion, a serious pattern in ocular surface epithelial disorder, has not been reported. Pharmacologic blockade of the cholinergic muscarinic receptors or surgical excision of the lacrimal glands to decrease tear secretion is known to be a useful technique to desiccate the ocular surface in animal models.24,30 However, these techniques have some disadvantages in assessing the critical function of ocular surface disorder, because it is difficult to exclude the complex influence of surgical insult or pharmacologic alteration after neural stimulation in the lacrimal gland. In contrast to these established models, however, the treatment technique we used to desiccate the ocular surface—namely, exposing rat eyes to continuous low-humidity air flow—is minimally invasive and provokes no pharmacological alteration in neural stimulation of the lacrimal gland. Moreover, our results of serum application in our rat model are in agreement with the successful clinical results of applying autologous serum to the ocular surface of individuals afflicted with dry eye.1,2,7 These findings, taken together, show that this model could be applicable in the investigation of useful drugs for dry eye.

Consistent with previous reports,20,21 our study provides evidence of a strong relationship between the pathogenesis of corneal epithelial disorder and induction of apoptosis on the ocular surface of dry eye. However, the mechanism involved in drastically aggravated corneal epithelial erosion accompanied by an increase in apoptotic epithelial cell death was not clear from this experiment. It is well accepted that the obvious role of tear fluid is to provide essential factors, such as growth factors, vitamins, and nutrition to maintain homeostasis of the ocular surface epithelium or to promote differentiation, migration, and proliferation during the epithelial wound-healing process.33–35 The lack of a tear fluid component may compromise ocular surface integrity or epithelial reconstruction. During the corneal epithelial wound-healing process, cytokines triggering epithelial cell apoptosis by IL-1, IL-6, and TNFα have been expressed on epithelial cells or tear fluid.36–38 In our model, it is presumed that cytokines may be present on the ocular surface at increased levels, because the tear fluid condensed due to accelerated tear evaporation rates. It could be speculated, therefore, that a synergic effect between enhanced apoptotic signals and insufficient supplementation of essential factors to the corneal epithelia may have been involved in the pathogenesis of corneal epithelial erosion.

**FIGURE 6.** Effect of topically applied HBA and rat serum on corneal epithelial erosion induced by ocular surface desiccation. Eye drops (PBS and HBA or rat serum) were applied to both eyes. Drops were applied at 1-hour intervals during ocular surface desiccation. After 12 hours, quantitative analysis of the fluorescein-stained area of the corneal epithelium was performed on each eye. Serum application (A), HBA application (B). Data represent the mean ± SD of 12 experiments. *P < 0.05, **P < 0.01, ***P < 0.005, versus PBS treatment (paired Student’s t-test).

**FIGURE 7.** Micrographs of a representative pattern of rat corneal epithelial erosion treated topically with HBA or serum. After 6 hours of ocular surface desiccation, rats’ eyes were fixed, and the cross-sectioned corneas were stained with hematoxylin and eosin. PBS (A), 20% serum (B), 80 mM HBA (C), 100% serum (D). Magnification, ×100.
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We demonstrated that both serum and HBA significantly prevented the appearance of corneal epithelial cell degeneration and suppressed apoptotic cell death due to ocular surface desiccation. Based on the data that the endogenous concentration of HBA in rat serum used in this study was negligible compared with the effective dosage, the protective effect of serum may be mediated by other serum components that are essential in maintaining ocular surface homeostasis. Recently, Higuchi et al. established a number of serum components that contribute to the suppression of cultured human conjunctival cell apoptosis induced by serum deprivation (Higuchi A, et al. IOVS 2002;43:ARVO E-Abstract 3171). They reported that apoptosis was suppressed by several components of serum, such as growth factors, vitamins, and serum proteins, and that these antiapoptotic effects act synergically. There may be some overlapping pivotal event between HBA and serum components responsible for the improvement of ocular surface epithelial disorder, although the exact mechanism involved remains unclear.

A recent study reported that HBA causes a significant reduction in the mitochondrial nicotinamide adenine dinucleotide (NAD) couple, which was reflected by an increase in mitochondrial membrane potential, in the working perfused rat heart. In another study, HBA showed a neuroprotective effect against mitochondrial dysfunction induced by mitochondria reduced nicotinamide adenine dinucleotide dehydrogenase (NADH) multienzyme complex inhibitor, which decreases cell respiration and mitochondrial proton pumping and increases free radical production, in cultured mesencephalic neurons. We suspect that a possible mechanism involved in the antiapoptotic effect of HBA is the suppression of multiple types of apoptosis signals induced by ocular surface desiccation, by preventing proapoptotic factor release by protecting the mitochondrial membrane potential. However, further study is needed to clarify the critical events involved in the suppressive effects of HBA on apoptosis.

In this study, topically applied HBA showed no adverse effects on the rat ocular surface. In addition, an intravenous dose toxicity test repeated at 4 weeks in dogs and rats showed no observable adverse effects on both systemic and ocular tissues at a dosage of a 400 mg (3.17 mM)/kg per day (data not shown). Taking these data together, we have shown a protective effect of HBA against corneal epithelial disorder in a tear-deficiency model, suggesting the potential usefulness of HBA in the clinical treatment of ocular surface epithelial disorders in patients with dry eye. However, we have not established the effect on mild types of dry eye, such as keratoconjunctivitis sicca, superficial punctate keratopathy, and rose bengal staining on the conjunctiva. Further investigations are now under way to clarify the effects on chronic symptoms of dry eye and the critical mechanisms involved.

References


