Effect of Topically Applied Na-Hyaluronan on Experimental Corneal Alkali Wound Healing

Jang-Hyun Chung M.D., Ph.D., Hyung-Joon Kim, M.D.,* Per Fagerholm, M.D.,* and Byung-Chae Cho, M.D.

Departments of Ophthalmology, Mokdong Hospital and Medical Research Center, Ewha Womans University, Seoul, Korea and *St. Erik's Eye Hospital, Karolinska Institute, Stockholm, Sweden

The effect of topically applied 1% sodium hyaluronate (Na-HA) on the healing of a standardized corneal alkali wound was studied. The healing of the epithelium, stroma, and endothelium was evaluated separately, using quantitative methods. Central corneal alkali wound was produced in one eye of the rabbits by applying a 5.5-mm round filter paper, soaked in 1 N NaOH, for 60 seconds. 1% Na-HA in the treatment group and phosphate buffered saline (PBS) in the control group were given topically 4 times per day for 2 days, 1- and 3-weeks. Epithelial and endothelial healing was assessed morphometrically from standardized photographs and micrographs, respectively. Stromal healing was determined by counting polymorphonuclear leukocytes (PMN) and keratocytes in the central and marginal wound areas. A positive healing influence was observed in the epithelium. In stromal healing, 1% Na-HA treated corneas showed less PMNs and more keratocytes than the control group, suggesting that topically applied 1% Na-HA may suppress the stromal PMN infiltration and enhance the keratocyte repopulation during corneal alkali wound healing. However, no significant difference was found in morphometric evaluation of endothelial healing between the two groups.

Key words: hyaluronan, cornea, alkali wound, epithelium, stroma, keratocyte, PMN, endothelium, rabbit

INTRODUCTION

Corneal alkali burns cause a severe ocular damage. Corneal wound healing following alkali burns is a long-term process with various complications such as neovascularization, dense opacity, melting and perforation. Many therapeutic trials with enzyme inhibitors, antiinflammatory agents, anticoagulants, mechanical barriers, oxygen radical scavengers, immunosuppressors, and various healing promoters have been used to manage this clinically devastating disorder with limited success.

Sodium hyaluronate (Na-HA) is a viscoelastic substance which does not induce inflammation. It is synthesized in the cell membrane and has been reported to be involved in cell protection, control of cell migration, growth control, cell differentiation, and tissue morphogenesis. It is widely distributed in connective tissues as an important constituent of the extracellular matrix. In previous experiments, the effect of topically applied Na-HA was examined...
in a standardized corneal alkali wound healing model and its positive effects on healing were reported.\textsuperscript{15} However, the mechanisms by which topically instilled Na-HA influence corneal alkali wound healing have not yet been explained. Although various humoral and cellular factors are involved in the repair process, the main cellular components of stromal remodelling following corneal alkali burns are polymorphonuclear leukocytes (PMNs) and keratocytes, which contribute to either stromal collagen degradation, or extracellular matrix synthesis.\textsuperscript{8,19-21}

In the present study, the effect of 1% Na-HA on corneal alkali wound healing in the epithelium, stroma and endothelium was evaluated quantitatively. The aim of this investigation focussed mainly on the influence of topically instilled 1% Na-HA on PMN infiltration and keratocyte repopulation during stromal healing following alkali burns.

**MATERIALS AND METHODS**

**Injury and treatment**

All experimental procedures were in accordance with the ARVO Resolution on the Use of Animals in Research. 112 New Zealand white female rabbits, weighing 3 kg, were used. The corneal alkali wounds were produced by placing a round 5.5 mm filter paper, soaked in 1 N NaOH, on the central cornea for 60 seconds following intravenous sodium pentobarbital anesthesia. The cornea was then rinsed with balanced salt solution (BSS) for 2 minutes. One eye of each rabbit was used for the experiment. The details of this technique have been described previously.\textsuperscript{22} The wound size and intensity in this model was small enough to avoid melting or vascular ingrowth but large enough to inhibit spontaneous healing to transparency. 1% Na-HA (Healon®, M.W., $4 \times 10^6$, Kabi Pharmacia, Uppsala, Sweden) was instilled topically 4 times per day in the treatment group and phosphate buffered saline (PBS) in the control group.

**Epithelial healing**

The anaesthetized rabbits received one drop of 2% fluorescein solution (Smith & Nephew Pharmaceuticals Ltd, England) to stain epithelial defects. The corneas were examined by a Haag-Streit slit-lamp and photographed, using a Nikon MK10 camera, Micro Nikkor, 105mm, 1:4 objective, and a Kodak Wratten 47B filter. The fluorescein stained area was morphometrically evaluated from standardized photographs.\textsuperscript{22} In each cornea a linear regression coefficient for the initial epithelial healing rate was calculated. The defect areas were measured at 4 different time intervals, i.e. 6, 12, 30, and 36 hours. The initial epithelial healing rates were measured in 12 eyes treated with 1% Na-HA and compared with 12 corneas from the PBS group. The recurrent epithelial defect areas were measured every 4 days for 3 weeks. In order to perform statistical comparison for the effect of 1% Na-HA, the accumulated size of the epithelial defect areas in each cornea was calculated from 4 days to 3 weeks after the initial wound.

**Quantitative cell counting and morphologic examination**

Twelve animals in each treatment group were killed at different time intervals, i.e. 2 days, 1 week, and 3 weeks. Each cornea was fixed with 4% formaldehyde and dehydrated in a graded series of alcohol and then embedded in paraffin. 4-m sections were cut and stained with haematoxylin and eosin for light microscopic examination. Stromal cell count (PMNs and keratocytes) was performed both in the central and marginal areas of the alkali wounded cornea as shown in Figure 1. In brief, the whole histological specimen was photographed. The area for differential cytology was decided in the $\times 100$ magnified photographs. The line between the highest point of the anterior stromal surface and the peripheral margin of the retrocorneal membrane indicated the mid-line of the marginal area for differential cytology. The central area was decided from the middle of the paired marginal areas. Differential cytology was performed in blind manner in the area of the whole stromal thickness $\times 1.0$ mm width by light microscopy at $\times 400$ magnification. Two corneas in each treatment group were prepared for electron microscopy. The corneas were fixed with 4% glutaraldehyde at 4°C for 48 hours. The specimens were rinsed three times in PBS and dehydrated in graded alcohol and
propylene oxide, and post-fixed in 2% osmium tetroxide for 2 hours. After dehydration the specimens were embedded in epon, and thin sections were cut on an ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-600 transmission electron microscope.

Endothelial healing

Eight animals in each group were killed after 3 weeks treatment. The corneas were excised with a 1-mm scleral rim and immediately stained in alizarin red for 2 min. After rinsing, the specimens were immersed in 99% ethanol for 30 sec. and then stained in 0.25% trypan blue for 60 sec. Immediately after staining, the endothelium was examined in the light microscope. Standardized photographs were taken for morphometric evaluation of the endothelial defect area. One cornea in the PBS group developed neovascularization among 16 examined eyes and was excluded from the endothelial morphometry.

Statistical analysis

Statistical analysis of data was undertaken using Wilcoxon’s two sample rank test. The level of statistical significance used was 0.05.

RESULTS

Epithelial healing

There was no statistical difference in the initial epithelial wound size between controls and the 1% Na-HA treated group. The initial epithelial defect was completely re-surfaced during the first two days (Fig. 2). The initial epithelial healing rate in the 1% Na-HA treated group was significantly increased compared to the PBS group (Table 1). In the late healing phase, 1% Na-HA treated corneas showed smaller epithelial defect and better maintenance of the re-surfaced epithelium than those of control eyes (Fig. 3).

Stromal healing

Immediately after alkali damage, most of the keratocytes disappeared and were not discernable by light microscopy until 2 days (Fig. 2). After 2 days no statistical difference was observed in the number of either PMNs and keratocytes in the central cornea.

Fig. 2. Micrographs showing the central part of the re-surfaced corneal epithelium and the anterior stroma 2 days after alkali wounding and treatment with either 1% Na-HA (A), or PBS (B). Neither PMNs nor keratocytes are identified in either groups. The 1% Na-HA treated cornea shows better organization of the re-surfaced epithelium and less hydration of the surface cells (arrows) when compared with the PBS treated cornea. (H & E, × 300)
Table 1. The effects of hyaluronan on the initial epithelial healing following alkali wounds

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>initial wound size (mm²)</th>
<th>initial epithelial healing rates (mm²/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.B.S</td>
<td>12</td>
<td>27.9 ± 0.86</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>Na-HA</td>
<td>12</td>
<td>27.1 ± 0.67</td>
<td>0.94 ± 0.08</td>
</tr>
</tbody>
</table>

*: p < 0.05,  n: number of corneas, Each value is the mean and 95% C.I.

![Graph showing cumulative size of epithelial defect area](image)

**Fig. 3.** Graph showing the cumulative size of the epithelial defect area in the individual corneas from the 1% Na-HA treated group and the PBS treated group. Each value is based on five observations from the 4th to the 21st day following alkali wounds. The mean of each group is indicated by a bar. Statistical comparison was performed using Wilcoxon's two sample rank test. p < 0.05.

between the treated and the control groups. The number of keratocytes in the marginal cornea was significantly higher in the 1% Na-HA treated eyes (Table 2). Morphological appearances of keratocytes at 2 days are shown in Figure 4. After 1 week, the number of stromal PMNs were significantly lower, both in the central and in the marginal corneas. However, no statistical significance was found in the number of stromal keratocytes either in the central or in the marginal areas (Table 3). The ratio of PMNs to keratocytes (%) in each specimen was determined. In the 1% Na-HA treated group, statistically significant reduction of the ratio was assessed in the central cornea at 1 week and 3 weeks and in the marginal cornea at 2 days and 1 week specimens (Tables 2, 3, 4). Extracellular matrix destruction and collagen disorganization appeared more severe around PMNs (Fig. 5) than keratocytes.

**Endothelial healing**

After 3 weeks, the endothelial healing was examined. 1% Na-HA treated corneas had smaller defects when compared with control eyes. The endothelial defect areas in the 1% Na-HA treated group and the PBS group were 5.68 2.37 mm² and 8.04 2.50 mm², respectively. However, there was no statistically significant difference between the two groups (Fig. 6, p > 0.05).

**DISCUSSION**

We have previously described a rabbit corneal alkali wound model which can be used for quantitative investigation of the epithelial, stromal, and endothelial healing.22-24 One important finding was that the healing of one layer influenced that of the other layers. Moreover, two phases in the repair process were discerned, i.e. an initial short-term

Table 2. Number of stromal PMNs and keratocytes, and ratio of PMNs to keratocytes (%) in each cornea at two days.

<table>
<thead>
<tr>
<th></th>
<th>Center</th>
<th>Margin</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMN</td>
<td>Keratocyte</td>
<td>P/K (%)</td>
<td>PMN</td>
<td>Keratocyte*</td>
<td>P/K (%)</td>
</tr>
<tr>
<td>P.B.S</td>
<td>10</td>
<td>0 (0-5)</td>
<td>0 (0-2)</td>
<td>–</td>
<td>59.5 ± 27.4</td>
<td>314.5 ± 39.0</td>
</tr>
<tr>
<td>Na-HA</td>
<td>10</td>
<td>0 (0-4)</td>
<td>0 (0-5)</td>
<td>–</td>
<td>35.2 ± 11.7</td>
<td>384.7 ± 47.4</td>
</tr>
</tbody>
</table>

*: p < 0.05,  †: p < 0.01, Parenthesis indicates the range of all number, Each value is the mean and 95% C.I.
Fig. 4. Electron micrographs of the central stroma in the 1% Na-HA treated cornea 1 week following alkali wounding. A dead keratocyte (A) and an activated keratocyte (B) are discernible in the stroma. Many cytoplasmic granules are dispersed in the extracellular matrix (arrow) and marked disorganization of collagen lamellar structure (asterisk) is around the dead keratocyte. (× 10000)

Table 3. Number of stromal PMNs and keratocytes, and ratio of PMNs to keratocytes (%) in each cornea at one week.

<table>
<thead>
<tr>
<th></th>
<th>Center</th>
<th></th>
<th>Margin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>PMN*</td>
<td>Keratocyte</td>
<td>P/K (%)*</td>
<td>PMN*</td>
</tr>
<tr>
<td>P.B.S.</td>
<td>10</td>
<td>185.2 ± 95.7</td>
<td>191.9 ± 76.5</td>
<td>52.5 ± 7.8</td>
</tr>
<tr>
<td>Na-HA</td>
<td>10</td>
<td>59.6 ± 13.5</td>
<td>239.7 ± 68.3</td>
<td>28.1 ± 9.5</td>
</tr>
</tbody>
</table>

*: p < 0.01,  Each value is the mean and 95% C.I.

Table 4. Number of stromal PMNs and keratocytes, and ratio of PMNs to keratocytes (%) in each cornea at three weeks.

<table>
<thead>
<tr>
<th></th>
<th>Center</th>
<th></th>
<th>Margin</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>PMN</td>
<td>Keratocyte</td>
<td>P/K (%)*</td>
<td>PMN</td>
</tr>
<tr>
<td>P.B.S.</td>
<td>10</td>
<td>67.32 ± 6.8</td>
<td>662.6 ± 167.7</td>
<td>9.4 ± 2.3</td>
</tr>
<tr>
<td>Na-HA</td>
<td>10</td>
<td>42.4 ± 18.3</td>
<td>734.3 ± 99.3</td>
<td>5.7 ± 2.2</td>
</tr>
</tbody>
</table>

*: p < 0.05,  Each value is the mean and 95% C.I.

healing phase and a long-term, late healing phase. The late stromal and endothelial healing began 1 week after the initial wound and reached the worst state at 3 weeks. The damaged epithelium resurfaced at about 2 days after wounding. Therefore, the choice of time intervals examined in this investigation, 2 days, 1 week and 3 weeks, are based on the natural repair process of the corneal alkali wound healing.

HA, a disaccharide polymer capable of binding considerable amounts of water, is one important constituent of the stromal extracellular matrix which appear to be involved in the expansion of tissue during wound healing that in turn allows cell migration. In alkali wounded eyes and other ocular surface disorders, different concentrations of Na-HA have been applied either clinically and experimentally. It has been reported that Na-HA provided better protection of the corneal epithelium, stimulated corneal epithelial migration, enhanced basal cell morphogenesis and thus provided an accelerated epithelial healing and reduced other secondary ocular surface problems. Our results also suggest that 1% Na-HA enhanced
Fig. 5. Electron micrograph showing PMN (arrow) in the central stroma of 3 weeks following alkali damage. Electron dense amorphous particles (arrow head) and severely disorganized lamellar structure (asterisk) are observed around a PMN which contained various sizes of vacuoles (V) in the cytoplasm, nucleus (N), phagosomes (L), and pseudopodia (P). (× 45000, inset: × 5000)

Fig. 6. Graph showing the size of the endothelial defect area 3 weeks after alkali wounding. Bars indicate the mean of each group.

wound healing following alkali damage.

In previous experiments, 1% and 2% Na-HA were applied in this experimental model, and the results were relatively positive at a certain time interval. However, the experiment left the question how topically applied Na-HA can influence on the stromal and endothelial healing. Several cell types, such as the corneal epithelium, keratocytes, endothelial cells, iris pigmented epithelium, lens epithelium were postulated to be capable of producing endogenous HA.16-18 In this investigation, the Na-HA treated cornea had, in general, smaller endothelial defect than PBS treated group. However, no statistically significant difference was found between the two groups. Recently, endogenous HA was observed in the various diseased human corneas, such as dystrophies, keratoconus, infection, bullous keratopathy, and trauma including alkali wounded human corneas.19 It was furthermore reported reactively formed HA in the rabbit cornea following alkali wounds. Formation of HA was observed in the healing epithelium, surrounding the repopulated keratocytes, and in the lacunae of the swollen stroma. A peak concentration of endogenous HA was determined 4 weeks after initial alkali damage.19 The role of the endogenously produced HA on corneal wound healing process is not known as yet. Further investigation should be needed for the evaluation of biological interrelation between the early epithelial healing and provided a better maintenance of the re-surfaced epithelium.

The matrix metalloproteinases, i.e. stromelysin, gelatinase, and collagenase, are responsible for degrading the stromal extracellular matrix during corneal alkali wound healing. A wide variety of cell types, including fibroblasts (keratocytes) and PMNs, secretes these proteolytic enzymes.2-3, 22-24 Keratocytes normally synthesize protein, presumably collagen and the protein of the ground substance, proteoglycans. After wounding, these cells release lysosomal enzyme and contribute to stromal collagen and proteoglycan destruction. In the events following corneal injury, PMNs escape almost immediately from the limbal capillaries and are carried by blinking across the entire cornea in the tear film, whence they enter the wound.25 Additional PMNs reach the wound by migration through the stroma.21 These PMNs release proteolytic enzymes into the wound tissue, perhaps for the purpose of remodeling dead and damaged cell debris. The present results suggest that topically applied 1% Na-HA suppresses PMN infiltration and enhances keratocyte repopulation during stromal
topical HA application and endogenous HA production in corneal alkali wound healing.

REFERENCES


26. DeLuise, V. P. and Peterson, W. S.: The use of...
Fig. 5. Electron micrograph showing PMN (arrow) in the central stroma of 3 weeks following alkali damage. Electron dense amorphous particles (arrow head) and severely disorganized lamellar structure (asterisk) are observed around a PMN which contained various sizes of vacuoles (V) in the cytoplasm, nucleus (N), phagosomes (L), and pseudopodia (P). (× 45000, inset: × 5000)

Fig. 6. Graph showing the size of the endothelial defect area 3 weeks after alkali wounding. Bars indicate the mean of each group.

wound healing following alkali damage.

In previous experiments, 1% and 2% Na-HA were applied in this experimental model, and the results were relatively positive at a certain time interval. However, the experiment left the question how topically applied Na-HA can influence on the stromal and endothelial healing. Several cell types, such as the corneal epithelium, keratocytes, endothelial cells, iris pigmented epithelium, lens epithelium were postulated to be capable of producing endogenous HA. In this investigation, the Na-HA treated cornea had, in general, smaller endothelial defect than PBS treated group. However, no statistically significant difference was found between the two groups. Recently, endogenous HA was observed in the various diseased human corneas, such as dystrophies, keratoconus, infection, bullous keratopathy, and trauma including alkali wounded human corneas. It was furthermore reported reactivity formed HA in the rabbit cornea following alkali wounding. Formation of HA was observed in the healing epithelium, surrounding the repopulated keratocytes, and in the lacunae of the swollen stroma. A peak concentration of endogenous HA was determined 4 weeks after initial alkali damage. The role of the endogenously produced HA on corneal wound healing process is not known as yet. Further investigation should be needed for the evaluation of biological interrelation between

discussion...
topical HA application and endogenous HA production in corneal alkali wound healing.

REFERENCES


26. Deluis, V. P. and Peterson, W. S.: The use of