Citrate or Ascorbate/Citrate Treatment of Established Corneal Ulcers in the Alkali-Injured Rabbit Eye

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Immediate treatment of alkali-injured eyes with citrate or ascorbate has previously been shown to prevent corneal ulceration and perforation in the rabbit. Other experiments showed that while ascorbate treatment of established ulcers did not appear to lead to significant healing it did reduce perforations by prolonging the presence of descemetocoeles. In the present experiment with established ulcers, alkali injuries were created with 1 N NaOH in a 12 mm corneal well for 35 seconds. Eyes were entered into the study with anterior, middle or posterior ulcers. When compared to controls, 10% citrate (qV 1/2 hr) significantly reduced the deepening of anterior stromal ulcers while 10% ascorbate/10% citrate (qV 1/2 hr/qV 1 hr—30 min apart) showed only a trend toward reduction of these ulcers (14 hr of dropping). The demonstration of healing (total vascularization or no ulcer) is significant when comparing the control group (8.3%) to the citrate treated group (58.3%, 0.01 < P < 0.009), but not the ascorbate/citrate group (18.2%). Sixty-seven percent of anterior stromal ulcers in the control group progressed to descemetocoele or perforation, compared to 8.3% in the citrate treated (0.003 < P < 0.004), and 45.5% in the ascorbate/citrate treated group (not significant). While the numbers of ulcers entered as middle stromal were too few to analyze statistically, the reduced numbers of perforations and increased stability prior to perforation in both treatment groups suggest a positive effect by both citrate and ascorbate/citrate. Treatment of posterior stromal ulcers did not prevent the development of descemetocoeles and perforations in either treatment group; however, the numbers in this category were too few to analyze. We suggest that 10% citrate (qV 1/2 hr × 14 hr) might be an effective treatment for corneal ulcers resulting from alkali injury to the human eye. Invest Ophthalmol Vis Sci 29:1110-1115, 1988

Topical ascorbate has been shown to protect alkali-injured rabbit eyes from undergoing corneal ulceration and perforation when treatment was started immediately after the injury.1,2 Ascorbate treatment of established corneal ulcers reduced the incidence of perforations but not the total number of descemetocoeles and perforations.3 The findings of decreased aqueous humor ascorbate, scurbutic fibroblasts and poor collagen production in the cornea after an alkali injury to the eye suggested a local tissue scorbutis. It was postulated that the scurbutic state of new corneal fibroblasts could be reversed by exogenous ascorbate, thereby encouraging healing of the cornea by stimulating collagen production. The lessened effect of ascorbate in significantly altering the outcome of established corneal ulcers after alkali injury may be related to large collections of inflammatory cells in the ulcer site and a deficiency of repair fibroblasts.

Topical citrate also protects the rabbit cornea from ulcerations and perforations when used immediately after severe and extremely severe alkali injury.4,5 Polymorphonuclear leukocytes (PMNs) are the predominant cell type observed in the ulcerating cornea after alkali injury6,7 and in the latter case virtually the only cell type present up to 2 weeks after the extremely severe burn. Subsequent in vitro studies on purified PMN suspensions, treated with citrate, showed significant inhibition of the respiratory burst, enzyme release, phagocytosis and locomotion.8-10 Inhibition of the adherence of PMNs to nylon fiber columns by citrate may parallel the inhibition of PMNs to vascular endothelium in the blood vessels of the conjunctiva and limbal arcades.11 If topical citrate can inhibit most PMN activities in vivo then a positive effect should be evident in established corneal ulcers after alkali injury.

The present study was conducted to determine if established corneal ulcers respond favorably to topical citrate treatment and if combined ascorbate/citrate therapy has any additional effect.

Materials and Methods

General Considerations

Animals were maintained and treated in full compliance with the ARVO Resolution on the Use of Animals in Research. Fifty-five male and female New...
Zealand Dutch strain albino rabbits weighing between 2.0 and 2.5 kg were anesthetized with 9 mg/kg xylazine and 13 mg/kg ketamine HCl given intramuscularly. Topical proparacaine hydrochloride (0.5%) was administered to each eye followed by propothing for the alkali injury. A 12 mm-35 second 1 N NaOH injury to the cornea was produced using a plastic well, as previously reported. Erythromycin ointment (0.5%) was applied to each eye immediately after the injury and twice daily for the duration of the experiment.

Double-masked examinations of each rabbit were performed three times a week (Monday, Wednesday and Friday) with a binocular dissecting microscope and a slit lamp to determine the presence of corneal ulceration, perforation, vascularization or infection. Once an ulcer was established the rabbit was assigned medication based on a randomization for triplicates procedure with the restriction that both eyes of a rabbit always received the identical medication.

Criteria For Entry Into the Study

To qualify for entry into the study, anterior stromal ulcers had to be present for two successive examinations. Middle and posterior stromal ulcers were admitted into the study on the day observed. These criteria ensured that the corneal ulcers entered into the study were active and not transient. Using this protocol 61 of 110 eyes (55.5%) qualified for entry into the study. The mean day that ulcers were entered into the study was not significantly different between treatment groups or ulcer depths. Two eyes in the citrate-treated group became infected, leading to rapid perforation, and were subsequently removed from the study. Of the remaining eyes, 19 ulcers from 12 rabbits were entered into the control group, 20 ulcers from 14 rabbits in the citrate group, and 20 ulcers from 15 rabbits into the ascorbate/citrate group.

Solution Preparation

Ten percent solutions of citrate or ascorbate had to be prepared for two successive examinations. Middle and posterior stromal ulcers were admitted into the study on the day observed. These criteria ensured that the corneal ulcers entered into the study were active and not transient. Using this protocol 61 of 110 eyes (55.5%) qualified for entry into the study. The mean day that ulcers were entered into the study was not significantly different between treatment groups or ulcer depths. Two eyes in the citrate-treated group became infected, leading to rapid perforation, and were subsequently removed from the study. Of the remaining eyes, 19 ulcers from 12 rabbits were entered into the control group, 20 ulcers from 14 rabbits in the citrate group, and 20 ulcers from 15 rabbits into the ascorbate/citrate group.

Treatment Regimen

Animals received two drops of the appropriate solution in the cul-de-sac of each ulcerated eye on the hour and half-hour from 8:00 AM until 9:30 PM. Eyes in the ascorbate/citrate group received ascorbate or citrate on an alternating schedule every 30 min. All rabbits were killed after the final examination (day 71).

6.5% Citrate Study

In a separate experiment 6.5% citrate was substituted for 10% citrate both alone and in combination with ascorbate. The experiment was conducted in a fashion identical to that detailed for 10% citrate.

Statistics

The results were analyzed for significance using the Chi-square and student t-tests. Anterior ulcers were evaluated for survival of perforation by the Kaplan-Meier product-limit method and compared for significance by the Gehan's generalized Wilcoxon test.

Results

10% Citrate, 10% Ascorbate/10% Citrate, Control

The clinical endpoints observed for ulcers entered with depths of anterior, middle or posterior stroma are summarized in Tables 1–3. At the end of the experiment anterior ulcers, treated with citrate, showed significantly more corneas either totally vascularized or not ulcerated with a central avascular zone (0.01 < P < 0.009) than controls (Table 1). Citrate treatment also provided marked protection against the deepening of these ulcers when perfora-
tions were considered alone ($P < 0.001$) or combined with descemetoceles ($0.003 < P < 0.004$). While there were no significant differences between the ascorbate/citrate-treated and control groups, a trend toward protection against the deepening of ulcers was suggested by the lowered incidence of perforations and the increased duration of ulcers prior to perforation in the ascorbate/citrate group. The numbers of eyes entered into the study as middle stroma were too few to obtain a statistical analysis (Table 2). However, both experimental treatment groups showed fewer perforations and the ascorbate/citrate group showed a greater delay before perforation occurred than the control group. Table 3 illustrates the lack of any observed effect by citrate or ascorbate/citrate treatment on ulcers entered as posterior stroma; however, there was an insufficient number of eyes available for detailed comparisons by statistical analysis. There was a high incidence of perforations occurring in all groups at similar times.

Anterior ulcers were also evaluated by the Kaplan-Meier product-limit method to determine the cumulative proportion of eyes that survived perforation. Censored eyes represent eyes entered into the study relatively late which had not reached an endpoint by the end of the experiment.

**Table 2. Analysis of clinical observations for established mid-corneal ulcers at end of experiment**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Ulcers entered as middle</th>
<th>Endpoint</th>
<th>Ulcer duration mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>1 Anterior 3rd</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 Perforations</td>
<td>16.7 ± 4.1</td>
</tr>
<tr>
<td>Citrate treatment</td>
<td>4</td>
<td>1 No ulcer</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Posterior 3rd</td>
<td>49.0 ± 17.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Descemetocele</td>
<td>58</td>
</tr>
<tr>
<td>Ascorbate/citrate treatment</td>
<td>5</td>
<td>1 No ulcer</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Middle 3rd</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Descemetocele</td>
<td>49.0 ± 12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 Perforation</td>
<td>38</td>
</tr>
</tbody>
</table>

**Table 3. Analysis of clinical observations for established posterior corneal ulcers at end of experiment**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Ulcers entered as posterior</th>
<th>Endpoint</th>
<th>Ulcer duration mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>3 Perforations</td>
<td>12.3 ± 3.3</td>
</tr>
<tr>
<td>Citrate treatment</td>
<td>4</td>
<td>1 Middle 3rd</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 Perforations</td>
<td>13.7 ± 4.5</td>
</tr>
<tr>
<td>Ascorbate/citrate treatment</td>
<td>4</td>
<td>1 Total vascular-</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ization</td>
<td>3 Perforations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18.0 ± 7.0</td>
</tr>
</tbody>
</table>

**Fig. 1.** Anterior stromal ulcers were evaluated by the Kaplan-Meier method to determine the cumulative proportion of eyes that survived perforation. Censored eyes represent eyes entered into the study relatively late which had not reached an endpoint by the end of the experiment.

Significant increase over control. Citrate treatment was also significantly higher than ascorbate/citrate treatment ($0.02 < P < 0.05$).

Figure 2 illustrates the data obtained from each treatment group for anterior stromal ulcers with each point representing a consecutive examination. The entry day for each ulcer was designated as exam 0 to avoid artifactual shifts caused by ulcers entered on different days. The final examination (exam 27) occurred on day 71. All ulcers were not represented in the last few exams because some were entered late into the study (this is reflected in the decreasing "n" values). Each increasing depth of ulceration was assigned an increasing whole number from 0 to 5. The depth of ulceration in the control group progressively worsened from exam 0 to 27, while citrate treatment halted the progression of ulcers, as a group, from the beginning of treatment (designated as exam 0) until the end of the experiment (exam 27). When compared to controls, treatment with ascorbate/citrate provided an intermediate degree of protection against the deepening of anterior ulcers. However, by the end of the experiment (exam 27) most of the protective effect of ascorbate/citrate treatment was lost.

When anterior, middle and posterior corneal ulcers going on to perforation are considered together for each treatment group, then the statistical significance is as follows: citrate < control, $P < 0.001$; ascorbate/citrate < control, $0.02 < P < 0.05$. When descemetoceles are added to perforations, then only the citrate group is significantly lower than control ($0.002 < P < 0.003$).

During the course of this experiment it was observed that translucent areas appeared in the corneas...
Fig. 2. The progress of eyes entered as anterior corneal ulcers is detailed over the course of the experiment. Exams were done every 2 to 3 days. The entry day for each ulcer was designated as exam 0 while exam 27 equals day 71. The “n” values decreased toward the end of the experiment because of ulcers that were entered into the study relatively late and had not come to a complete endpoint by the end of the experiment. Ulcers which totally vascularized (0) or perforated (5) before the end of the experiment continued to be included in the analysis of ulcer severity until the end of the experiment.

6.5% Citrate, 10% Ascorbate/6.5% Citrate, Control

The results of using 6.5% citrate with or without ascorbate are detailed in Table 4. The only statistical significance achieved in this experiment is a decrease in the total number of perforations for the experimental groups (citrate vs. control, 0.02 < P < 0.05; ascorbate/citrate vs. control, 0.02 < P < 0.05). If descemetoceles are combined with perforations then the differences are no longer significant.

Discussion

The protection which citrate provided against the deepening of established anterior corneal ulcers in the alkali-injured rabbit eye appears to be a clinically meaningful result. Citrate treatment significantly increased the number of corneas which ended the experiment totally vascularized or not ulcerated with a central avascular zone. It also significantly decreased the numbers of perforations alone and descemetoceles and perforations combined.

The favorable effect of citrate appears to be related to its ability to inhibit PMNs. Citrate has been shown to inhibit in vitro adherence of resting or augmented PMNs to nylon fiber columns as well as the locomotion of PMNs. PMNs are also inhibited from undergoing a respiratory burst, enzyme release or phagocytosis in the presence of citrate. Each of these functions can be activated by different mediators with varying sensitivities to divalent cations. The fact that citrate is a chelator of divalent cations and that this inhibition can be reversed by Ca<sup>2+</sup> or Mg<sup>2+</sup>
supports the contention that citrate is acting as a chelator of cations.

The therapeutic effect of topical citrate appears to diminish as the depth of the ulcer increases. Ulcers entered into the study with a depth of middle stroma showed a trend toward stabilization or healing but there were insufficient numbers of eyes to attain statistical significance. There were also insufficient data to statistically analyze posterior corneal ulcers, but no difference appeared to be present when compared to control. Although it is anticipated that significant inhibition of PMNs would have taken place in the bed of a posterior ulcer, under the influence of citrate, there may have been a cascade of events set in motion before citrate treatment began or not affected by citrate in these late phases. It is more logical to presume, however, that the prior accumulation of proteolytic enzymes or products from the dismutation of reactive oxygen species are sufficient to perforate the eye.

The more frequent appearance of translucent areas in corneas receiving citrate treatment confirms a previous observation from an extremely severe alkali burn model (4 N NaOH-45 seconds-12 mm). Although the corneal tissue appears thinner by slit-lamp microscopy the exact significance of this observation is unknown.

The use of citrate alternating with ascorbate topically produced a result intermediate between citrate alone and the control. The fact that the addition of ascorbate seemed to reduce the effectiveness of citrate may be more a function of halving the frequency of citrate, thus lowering the mean corneal citrate concentration, rather than any deleterious effect of ascorbate. This is suggested by the fact that reducing the concentration of citrate to 6.5%, but maintaining every one-half hour dosage, reduced the number of categories which were statistically significant to only the total number of perforations (10% citrate vs. control = 0.001, 6.5% citrate vs. control = 0.02 < P < 0.05). It is also not surprising, since in a previous study topical ascorbate reduced the frequency of perforation, without changing the total number of descemetoceles and perforations, in established corneal ulcers after alkali injury. The inability of ascorbate to meaningfully heal established corneal ulcers might reflect a deficiency or incapacitation of fibroblasts. Alternately, ascorbate may have worsened established ulcers by enhancing PMN activity in the ulcer bed not completely suppressed by citrate. This latter possibility seems unlikely in the alkali-injured eye because hourly ascorbate treatment did not worsen established ulcers in the previous study. The efficacy of hourly citrate drops (10%) on established ulcers has not been tested and remains unknown.

The effect of ascorbate on PMNs is very controversial. Phan et al showed that ascorbate appeared to promote corneal ulceration in an ocular thermal burn model where intraocular ascorbate levels were normal. This unfavorable response is thought to be caused by the activation of PMNs which then enter the injured cornea in increased numbers and release hydrolytic enzymes, giving rise to a corneal ulcer. From these results it has been concluded that ascorbic acid treatment might be contraindicated in ocular injuries which are not typified by scurvy fibroblasts. Alternately, the use of ascorbate as an antiinflammatory agent has been suggested after certain experiments appeared to show that ascorbate might actually inhibit PMNs. Ascorbate is clearly not as helpful as citrate in established corneal ulcers, apparently because few fibroblasts are present in the ulcer bed while citrate-inhibitable PMNs abound.

Topical citrate appears to be effective not only in the prevention of aseptic ulceration after alkali injury in the rabbit eye but also in the treatment of anterior corneal ulcers resulting from such injury. Since this favorable effect seems to result from inhibition of PMNs it is likely that citrate would have a similar effect in the human. This expectation is also based on our in vitro studies showing the inhibition of human PMNs. Results from the national clinical trial of ascorbate and citrate, used separately, in alkali-injured human eyes will help to answer some of these questions. The utility of each foodstuff in septic ulceration of the cornea also remains an unanswered question.

Key words: citrate, ascorbate, corneal ulcers, alkali burns, rabbits

Acknowledgments

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References

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