The Efficacy of Topical Citrate after Alkali Injury Is Dependent on the Period of Time it Is Administered

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The neutrophilic response in the alkali-injured rabbit cornea can be separated into two peaks: the first from 12-24 hr and the second at 21 days. In the experiments detailed here, alkali-injured rabbit eyes were treated for 0-7 days (early peak), 6-35 days (late peak), or 0-35 days (both peaks) with 6.5% or 10% topical citrate. Clinical evaluation of these eyes showed that treatment with 10% citrate (0-35 days) significantly reduced the incidence and severity of corneal ulceration from the control level. When 10% citrate was administered to block the early neutrophilic peak, the incidence of corneal ulceration was also significantly reduced (33.3% from 72.2%). The average ulcer depth increased gradually, reaching an intermediate level well below the control group by day 35. In contrast, 10% citrate treatment of the late peak had no significant effect on the incidence of ulceration; however, these ulcers were less severe than those in the control. When 6.5% topical citrate (0-35 days) was used, there was a significant reduction in the severity but not the incidence of ulceration. In addition, the beneficial effects obtained from separate treatment of the early and late PMN peaks were mostly lost. These experiments suggest that both the early and late peak of the PMN response after alkali injury are important to the development of corneal ulcers. We hypothesize that the early use of 10% citrate diminishes the initial peak of PMNs, interfering with the release of self-perpetuating inflammatory mediators, thereby decreasing the later accumulation of PMNs that cause ulceration. Invest Ophthalmol Vis Sci 30:1062-1068, 1989

Polymorphonuclear leukocytes (PMNs or neutrophils) play a major role in the development of corneal ulcers after alkali injury of the rabbit eye.\(^1\)\(^-\)\(^3\) It has been reported that the initial neutrophil invasion is limited to the peripheral cornea, with a peak between 12 to 24 hr, subsiding by day 3.\(^4\) A second, much greater neutrophilic infiltration, began about day 10, rising to a peak at day 21, and subsiding by day 28. There is a strong correlation between the second PMN invasion and the subsequent development of corneal ulceration. In the stroma of these corneas, stimulated neutrophils release their hydrolytic enzymes and undergo phagocytosis and a respiratory burst to generate free oxygen radicals. These metabolic products favor the further destruction of collagen and glycoproteins in the cornea.

Topical citrate, administered immediately after an alkali injury and continued until the end of the experiment, significantly reduced the incidence of corneal ulceration and perforation in rabbit eyes.\(^5\)\(^-\)\(^6\) In these and other studies, citrate was noted to prevent the accumulation of PMNs in the cornea.\(^4\)\(^5\)\(^-\)\(^7\) Citrate prevents PMN accumulation, probably by inhibiting most PMN functions, including adherence to the vascular endothelium, directed locomotion through the corneal stroma, and their metabolic stimulation.\(^8\)\(^-\)\(^11\) By reducing the numbers and activity of PMNs in the injured cornea the destructive process leading to ulceration is diminished.

The purpose of this study was to determine the clinical effect of topical citrate on the early peak and, separately, the late peak of PMNs. A dose-response relationship was established by independently treating with 6.5% or 10% citrate. The presence, time of development, severity and healing of corneal ulceration were the parameters used to judge the clinical response.

**Materials and Methods**

**General Considerations**

Animals were maintained and treated in full compliance with the ARVO Resolution on the Use of Animals in Research. Female New Zealand Dutch strain albino rabbits weighing between 2.0 and 2.5 kg were anesthetized with intramuscular xylazine (9 mg/kg) and ketamine HCl (13 mg/kg). Two drops of proparacaine hydrochloride (0.5%) were given to...
The continuous citrate group was administered 10% citrate every hour throughout the experiment. The final pH of each solution was brought up to 7.3 with HCl and the volume was increased to 100 ml with Adsorbotear. These solutions were prepared fresh at the beginning of each experiment and kept refrigerated.

Preparation of Citrate Solution

Citrate (10%) was prepared by dissolution of 15.313 g of trisodium citrate in Adsorbotear without ethylenediamine tetraacetic acid (EDTA). Absorbotear was prepared in our laboratory according to instructions from Alcon (Fort Worth, TX), except for the omission of EDTA. Ten grams of trisodium citrate were added to the Adsorbotear to achieve a concentration of 6.5% citrate. The final pH of each solution was adjusted to 7.3 with HCl and the volume was brought up to 100 ml with Adsorbotear. These solutions were prepared fresh at the beginning of each experiment and kept refrigerated.

Treatment Regimen for 10% Citrate Experiment

Animals received two drops of the appropriate solution in the lower cul-de-sac of each eye on the hour from 8:00 AM until 9:00 PM. The control group was given Adsorbotear drops for the entire experiment. The continuous citrate group was administered 10% citrate drops for the entire experiment. The early citrate treatment group received 10% citrate drops for the first seven days and at 8:00 AM on the eighth day they began receiving Adsorbotear, continuing throughout the rest of the experiment. This treatment regimen was designed to prevent the early influx of PMNs, but not influence the late peak. The late citrate group received Adsorbotear drops for the first 5 days and at 8:00 AM on the sixth day began receiving 10% citrate drops throughout the duration of the experiment. This late citrate treatment was designed to allow the initial invasion of PMNs to occur, but to prevent the subsequent accumulation of PMNs that produce the late peak.

Forty rabbits, ten (20 eyes) per group, were entered into this experiment. One eye of one rabbit, belonging to the late citrate treatment group, was not alkali-injured because of the presence of a congenital anomaly. One rabbit (two eyes) from the early citrate treatment group died on day 2 and one rabbit (two eyes) from the control group died on day 9. None of these eyes had ulcerated at the time of death of the rabbit.

Treatment Regimen for 6.5% Citrate Experiment

In a separate, but identically conducted experiment, 6.5% citrate was substituted for 10% citrate. Forty-two rabbits were entered into the study (11 control—22 eyes; 11 continuous citrate—22 eyes; ten early citrate—20 eyes; and ten late citrate—20 eyes).

Statistics

Certain results were evaluated by calculating the mean ± standard error of the mean. The results were analyzed for significance using the Chi-square and student t-tests.

Results

Timed Administration of 10% Citrate

Figure 1 illustrates the severity of ulcers in each 10% citrate treatment group. Control eyes ulcerated to an average depth of 2.8 at day 35, while continuous citrate treatment resulted in an average ulcer depth of 0.5 by day 35. Throughout the experiment the average at any point in these two groups was significantly different.

The average ulcer depth in eyes from the early citrate treatment group (0–7 days) paralleled the continuous citrate group until day 19 at which time it increased to significantly deeper ulceration (P = 0.05). From that point on the severity of ulceration in the early citrate group continued to increase gradually, reaching an average ulceration depth midway between the control and continuous citrate groups by day 35.
Ulcer depth in eyes from the late citrate treatment group (6–35 days) paralleled the control group until day 16. Thereafter, the average depth of ulceration did not progress, also leading to an average ulcer depth midway between the control and continuous citrate groups at day 35. The leveling of this curve represents a balance between the significant healing of six eyes (0.001 < \( P < 0.01 \)) from a mean ulcer depth of 2.0 ± 0.5 to 0.3 ± 0.2 and the simultaneous deepening of other ulcers in the same group.

Table 1 is a summary of the clinical results and Table 2 compares the significance of the clinical observations from the 10% citrate treatment groups. Control eyes had a 72.2% incidence of ulceration; nine of these ulcers were categorized as descemetoceles or perforations. The incidence of ulceration in the continuous citrate group was significantly less (30.0%, 0.001 < \( P < 0.01 \)) with only one descemetocele developing (0.001 < \( P < 0.01 \) vs. descemetoceles plus perforations in the control group). The raw numbers show a strong shift from more severe, deeper ulcers in the control group to more superficial, shallow ulcers in the continuous citrate group.

The early citrate treatment group (0–7 days) showed a 33.3% incidence of ulceration; a significant reduction when compared to control eyes (0.001 < \( P < 0.01 \)). There were also fewer perforations in this early treatment group, but when combining desce-

**Table 1. Clinical results from 10% citrate experiment**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Continuous citrate</th>
<th>Early citrate treatment</th>
<th>Late citrate treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No ulcer</td>
<td>5</td>
<td>14</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>2. Ulcer depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Anterior</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>b. Middle</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>c. Posterior</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>d. Descemetoceles</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>e. Perforations</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>f. Descemetoceles and perforations</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>3. Total ulcers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. During experiment</td>
<td>13 (72.2%)</td>
<td>6 (30.0%)</td>
<td>6 (33.3%)</td>
<td>13 (68.4%)</td>
</tr>
<tr>
<td>b. Endpoint</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
<td>Same</td>
</tr>
<tr>
<td>4. Mean day of ulcer occurrence</td>
<td>17.0 ± 1.7</td>
<td>26.0 ± 3.6</td>
<td>17.5 ± 1.7</td>
<td>14.8 ± 1.2</td>
</tr>
</tbody>
</table>

* Four ulcers completely healed during the course of treatment.
Table 2. Significance of clinical observations from 10% citrate experiment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Compared to</th>
<th>Clinical endpoint* from Table 1</th>
<th>Significance† (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early citrate</td>
<td>Control</td>
<td>3a</td>
<td>0.001 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td>Early citrate</td>
<td>Continuous citrate</td>
<td>2a, 4</td>
<td>0.02 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td>Late citrate</td>
<td>Control</td>
<td>2b, 2f</td>
<td>0.02 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td>Late citrate</td>
<td>Continuous citrate</td>
<td>3a</td>
<td>0.01 &lt; P &lt; 0.02</td>
</tr>
<tr>
<td>Continuous citrate</td>
<td>Control</td>
<td>4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2e</td>
<td>0.02 &lt; P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2f, 3a</td>
<td>0.001 &lt; P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.01 &lt; P &lt; 0.02</td>
</tr>
</tbody>
</table>

* The clinical endpoint column refers to Table 1, not significant if absent. † Student t-test or Chi-square test.

metceles and perforations this difference was not significant.

The incidence of ulceration in the late citrate treatment group (6–35 days) was 68.4% during the experiment (not significant vs. control, 0.01 < P < 0.02 vs. continuous citrate) but by the end of the experiment (day 35) only 47.4% of these eyes were ulcerated. Significantly fewer descemetoceles and perforations occurred in this group when compared to control eyes (0.02 < P < 0.05).

When ulcers began, the average time of their appearance was not statistically different in the control, early citrate, or late citrate treatment groups (17.0, 17.5 and 14.8 days, respectively). In the continuous citrate group the average time of ulcer formation was day 26.0, significantly later than all other groups.

Timed Administration of 6.5% Citrate

Tables 3 and 4 show that the incidence of ulceration in the continuous 6.5% citrate (45.5%) and control (63.6%) groups were not statistically different. However, ulcers in the continuous citrate treatment group were much less severe, with a definite shift, toward shallow ulcers when compared to the high incidence of descemetoceles and perforations in the control group (0.001 < P < 0.01, descemetoceles plus perforations). The mean time of ulcer formation in the continuous citrate group was significantly delayed (P < 0.001).

Ulceration in the early citrate group was negligible until day 8, when citrate treatment was discontinued (Fig. 2). The average ulcer depth of this group then increased dramatically at day 10, equaling and then paralleling the control eyes from 12 days until the end of the experiment. In the late citrate group, ulcer development was initially stabilized but then progressed to control levels at the end of the experiment (day 35, Fig. 2).

Discussion

It has been reported that PMNs invade the peripheral rabbit cornea within hours after an alkali injury.
reaching maximum numbers between 12 and 24 hr. This small peak of PMNs subsided until about day 10 when the level of PMN accumulation rose substantially at day 14, peaking at day 21. In those experiments continuous 10% citrate treatment was found to inhibit the first peak by 63% and the second peak by 92%, minimizing corneal ulceration. The authors concluded that the initial PMN peak was probably not related to the pathogenesis of corneal ulceration, whereas the late peak did correlate with the development of ulceration.

The results presented in the current paper suggest that the early peak of PMNs is the linchpin for the development of corneal ulceration during the late PMN peak. This statement is supported by the finding that the incidence of corneal ulceration was significantly reduced in the group treated with 10% citrate for only the first week. This probably results from the reduced early accumulation of PMNs in the cornea, where they would release self-regulating mediators that recruit many more PMNs. This inhibition of the first peak of PMNs by citrate would therefore indirectly reduce the late peak by blocking the amplifying effect of mediators on the inflammatory process.

It is presumed that the lag period in citrate effect, from 6 to 19 days in the late citrate group, was caused by the inability of citrate to immediately abrogate the powerful physiologic processes associated with activated PMNs. By treating these animals after only 6 days, the first peak occurs, allowing these accumulated PMNs to release hydrolytic enzymes and free
oxygen radicals and to generate mediators capable of self-recruitment. The healing effect of citrate on corneal ulcers only became clear on day 19, when the apparent combined effect of inhibition of PMN adherence, locomotion and metabolic stimulation reversed the ulceration trend. The events occurring in this experiment are similar to the healing effect of topical 10% citrate noted in corneal ulcers that were established.

In the case of 10% citrate, the greater success of early over late treatment in reducing the incidence of corneal ulceration suggests that removal of the amplifying effect of the first PMN peak by citrate is of primary importance, playing a major role in inhibiting the second PMN peak.

There are other potential explanations for the favorable effect of early 10% citrate treatment on the subsequent formation of corneal ulcers. We are investigating the buffering capacity of citrate in reducing the destructive effects of prolonged exposure of collagen to low concentrations of alkali. This buffering effect might limit the release of PMN stimulant(s) from the corneal matrix. Citrate inhibition of the production of inflammatory mediators by cells, other than PMNs, removing the stimulus for the late PMN peak. Least likely, citrate might react with collagen, producing a stable complex, inaccessible to further destruction by PMNs.

The current and previous experiments clearly show that 10% topical citrate is superior for achieving the full benefit of citrate therapy. For the best results, PMNs must be prevented from entering the cornea immediately after the alkali injury and continuously blocked by citrate (10%) treatment until the end of the experiment. The use of 10% citrate in the current study showed a significant reduction in the incidence of ulceration (continuous treatment), a significant retention of the inhibitory effect on the severity of ulceration (early treatment), and a permanent healing of ulcers (late treatment) not observed for 6.5% citrate therapy. Based on the studies detailed here and those of Paterson et al., continuous treatment with 10% topical citrate probably reduced the early and the late peak of PMN invasion into the cornea.

The greater success of 10% over 6.5% citrate in the early treatment protocol may result from the greater inhibition of the first PMN peak (10% citrate = 63% inhibition of PMN accumulation, 7.5% citrate = 32% inhibition of PMN accumulation as compared to controls). We believe that when 10% citrate treatment was stopped on day 7, few PMNs were present, with little metabolic stimulation taking place in the cornea. Small molecular weight inflammatory mediators, produced from direct alkali injury of corneal proteins, would have diffused away, diminishing many of the chemotactic factors available to attract additional PMNs. In contrast, 6.5% citrate treatment for 7 days probably caused some PMN metabolic inhibition but failed to stop many PMNs from invading the cornea during the first peak. When the citrate suppression is removed at 7 days, these PMNs might then quickly respond with increased metabolic stimulation from the large, less diffusible stimulant(s) for the respiratory burst and hydrolytic enzyme release.

In a previous study, 5 or 10% citrate drops were applied to the rabbit eye after removal of the corneal epithelium. Citrate levels of 3.9 or 8.1 mM were obtained in the corneal stroma, respectively. Interpolation from these results suggests that corneal stromal citrate levels in alkali-injured eyes treated with 6.5% citrate would be approximately 5.2 mM. Citrate levels below 8 mM are probably in the low range for PMN inhibition. This is corroborated by topical citrate inhibition of the first PMN peak, which was found to be dependent on the concentration of citrate in the dropping solution (5% citrate = 0% inhibition, 7.5% citrate = 31.5% inhibition, 10% citrate = 62.6% inhibition). We conclude that the corneal citrate levels achieved, particularly in the anterior portion of the stroma, after dropping with continuous 10% citrate are probably high enough to strongly inhibit the cellular functions of PMNs.

The use of topical 10% citrate for alkali injuries of the human eye is not recommended until the results of the national randomized clinical trial are known. However, the findings of this study may have a significant impact on those results. It is clear from this paper that early and prolonged treatment of alkali-injured eyes with 10% citrate is the most promising approach to the prevention of or the reduction in the severity of corneal ulcers. If this treatment can be initiated on the first day of the injury it is possible that the stimulus leading to ulceration might be abrogated.

Key words: citrate, alkali injury, corneal ulceration, rabbit, polymorphonuclear leucocyte, inflammation

Acknowledgments

We gratefully acknowledge the technical assistance of Jonathan Preble in the performance of the experiments using 6.5% citrate.

References