EDTA Inhibits In Vitro Substrate Adherence Capacity of Macrophages: Endodontic Implications

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The disodium salt of ethylenediamine tetraacetate (EDTA) is a calcium ion chelator used in endodontics to enlarge root canals. This study investigated the effect of EDTA on substrate adherence capacity of rat inflammatory macrophages to determine if EDTA leakage to periapical tissues during root canal therapy can alter macrophage function. Inflammatory macrophages were obtained from Wistar rats and resuspended in RPMI-1640 medium. Substrate adherence capacity assays were carried out in Eppendorf tubes for 15 min of incubation at 37°C in a humidified atmosphere of 5% CO₂. The adherence index (AI) was calculated. Results showed that EDTA decreased substrate adherence capacity of inflammatory macrophages in a time and dose-dependent manner. The lowest EDTA concentration that caused a significant inhibition of AI was 50 mM (p < 0.05), and the EDTA concentration that caused half-maximal inhibition (IC₅₀) was 194 ± 20 mM (p < 0.01). Calcium chloride (10 mM) increased the adherence index of macrophages by 17.1% (p < 0.05) and decreased the EDTA inhibitory effect on AI by 49.5% (p < 0.05). We conclude that an EDTA concentration lower than that used in endodontics decreased the substrate adherence capacity of macrophages significantly. Adhesion is the first step in the phagocytic process and in antigen presentation, but leakage of EDTA to periapical tissues during root canals preparation may inhibit macrophage function and reduce periapical inflammatory reactions.

INTRODUCTION

The disodium salt of ethylenediamine tetraacetate (EDTA) was introduced to endodontics on the basis of its chelating effect on calcium ions (1). EDTA is used in endodontic therapy to facilitate canal identification and enhance chemomechanical enlargement of canals (2, 3, 4). Moreover, the use of EDTA in root canal preparation aids in cleaning and disinfecting the dentinal wall and preparing it for a better adhesion of filling materials (5). Finally, it causes increased dentin permeability, which enhances the action of drugs (6).

For an effective use of EDTA it must be applied with thin files, introducing it into root canal as deep as feasible (3). Therefore, leakage of EDTA to periapical tissues during root canals preparation is possible.

On the other hand, it has been demonstrated that inflamed periapical tissues contain a variety of immunocompetent cells, with macrophages predominating (2, 7). Macrophages are implicated in bone resorption (8) and play an essential role in the pathogenesis of human periapical pathosis (2, 9).

Macrophages, elicited nonimmunologically with caseinate or glycogen by washing the peritoneal cavity of rats with 0.9% NaCl 4 to 5 days after intraperitoneal injection of 5 ml of 6% sodium caseinate, are described as “casein elicited macrophages” or “inflammatory macrophages” (10, 11). Such cells have many chemical and biochemical characteristics similar to those specifically activated by immunologic mechanisms in vivo (10). Although molecular mechanisms involved in macrophage function are poorly defined (12), it is well known that adherence is the first step in the phagocytic process of inflammatory macrophages (13).

Clinical observation of approximately 200 patients treated endodontically with 10% EDTA as an irrigant suggested that this drug produced no deleterious effect and that it is a valuable adjunct to the endodontist’s armamentarium (14). Thus, it has been thought that apical extrusion of EDTA during root canals preparation caused only a decalcifying action on periapical bone that was reversed in 3 to 4 days (2, 3). However, EDTA has been shown to act as an inflammatory stimulus in the dorsal muscle of albino rats (14) and to inhibit the binding of vasoactive intestinal peptide (VIP) to macrophage membranes (15).

The effect of EDTA on substrate adherence capacity of inflammatory macrophages was studied to determine if EDTA leakage during root canals preparation could modulate macrophage function altering inflammatory reactions in periapical tissues.

MATERIALS AND METHODS

The disodium salt of EDTA, calcium chloride, and RPMI-1640 medium were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Male Wistar rats aged 6 to 12 weeks were maintained on a 12-hr light/dark cycle and allowed free access to food and water. Utmost
precautions were taken so that the animals remained free from infection by environmental pathogens. Each rat was injected intraperitoneally with 5 ml of sterile 6% sodium caseinate. Animals were killed after 4 days by decapitation and the peritoneal cavity was washed with 10 ml of cold 0.9% NaCl. After a 2-min massage, the cell exudate was removed with a syringe and centrifuged for 10 min at 250 × g at 4°C. The contaminating red blood cells were lysed with cold 0.2% NaCl. The remaining cells were then washed with 0.9% NaCl by centrifugation, resuspended in RPMI-1640 medium, counted, adjusted in the same medium at 2–4 × 10^6 macrophages/ml, and, immediately, used for experiments. Mean cells per rat varied from 20 to 30 × 10^6, of which 85 to 95% were macrophages by morphological criteria in Giemsa and Papanicolaou staining techniques. Viability, as determined by trypan-blue exclusion, was always greater than 95%.

The quantification of substrate adherence capacity was carried out according to the technique described previously by De la Fuente et al. (16) with minor modifications. Aliquots of 180 µl of cell suspension were dispensed in Eppendorf tubes, which resemble the adherence to tissues as reported by Noga et al. (17) and De la Fuente et al. (16). EDTA (20 µl) was added to reach a final concentration ranging from 2 mM to 300 mM. Medium (20 µl) was added instead of EDTA to control samples. Adherence assays were performed at 5, 15, or 30 min of incubation at 37°C in a humidified atmosphere of 5% CO₂. After gentle removal (5 s in the vortex in position 5) of nonadherent cells, aliquots of 10 µl from each sample were taken and the number of nonadherent macrophages/ml was counted in Neubauer chambers. No agglutination of macrophages was observed. When two substances were tested simultaneously, 10 µl each were added together with 180 µl of cell suspension to the Eppendorf tubes, to a final volume of 200 µl. The adherence index (AI) was calculated according to the following equation:

\[
\text{A.I.} = 100 - \frac{\text{Nonadherent macrophages/ml}}{\text{Initial macrophages/ml}} \times 100
\]

All values were expressed as the mean ± SEM of the number of experiments, performed in duplicate. The data were evaluated statistically by Student’s t-test. A value of \( p < 0.05 \) (two tailed) was considered statistically significant.

**RESULTS**

EDTA inhibited the substrate adherence capacity of macrophages in all conditions tested. The inhibitory effect of EDTA was a time-dependent and dose-dependent phenomenon. The AIs obtained in control peritoneal macrophages and incubated with different EDTA concentrations (5 mM, 100 mM, and 300 mM) for 5, 15, and 30 min are shown in Fig. 1. As can be seen, the substrate adherence capacity of control macrophages increased progressively, reaching a maximum between 15 and 30 min. When 5 mM EDTA was added to the incubation medium no significative changes were found (\( p > 0.05 \)). On the other hand, higher EDTA concentrations decreased the AI significantly at all times tested. At 5 min incubation 100 mM and 300 mM EDTA decreased AI by 51.3% and 65%, respectively (\( p < 0.01 \)). At 15 min incubation the inhibitory effect of EDTA was greater still, persisting until 30 min. In subsequent experiments, incubation of cells was performed at 37°C for 15 min in a humidified atmosphere of 5% CO₂, to provide a maximal inhibitory effect of EDTA.

![Fig 1. Time course of EDTA-inhibition of AI of inflammatory macrophages.](image1)

**Fig 1.** Time course of EDTA-inhibition of AI of inflammatory macrophages. Macrophages (2–4 × 10⁶/ml) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in the absence (○) or presence of different concentrations of EDTA (5 mM; 100 mM; 300 mM). After 5, 15, or 30 min, reaction was stopped and the AI calculated. Each point is the mean of three separate experiments performed in duplicate.

![Fig 2. Concentration-effect curve for the effects of EDTA on AI of inflammatory macrophages.](image2)

**Fig 2.** Concentration-effect curve for the effects of EDTA on AI of inflammatory macrophages. Macrophages (2–4 × 10⁶/ml) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in the absence (control) or presence of increasing concentrations of EDTA (from 5 mM to 300 mM). After 15 min the reaction was stopped and the AI calculated. Each point is the mean of three separate experiments performed in duplicate.

The concentration-effect curve for the effects of EDTA on the AI of macrophages is shown in Fig. 2. The lowest EDTA concentration that caused a significant inhibition of AI was 50 mM (\( p < 0.05 \)) and half maximal inhibition (IC₅₀) was obtained at 194 ± 20 mM (\( p < 0.01 \)).

Incubation of cells with 5 mM calcium chloride alone increased AI of macrophages by 7.7% (\( p > 0.05 \)) (Table 1). Calcium chloride at 10 mM increased AI of macrophages by 17.1% (\( p < 0.05 \)). When 100 mM EDTA was added to the incubation medium together with 5 mM calcium chloride (CaCl₂) the inhibitory effect of
Table 1. Modulation by calcium chloride of the EDTA-effect on substrate adherence capacity of inflammatory macrophages

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (M)</th>
<th>AI Increment</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>71.4 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5 × 10⁻³</td>
<td>76.5 ± 5.7</td>
<td>+7.7% &lt;0.05</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10⁻²</td>
<td>63.6 ± 6.9</td>
<td>+17.1% &lt;0.05</td>
</tr>
<tr>
<td>EDTA</td>
<td>10⁻¹</td>
<td>39.3 ± 3.2</td>
<td>−45.0% &lt;0.01</td>
</tr>
<tr>
<td>EDTA</td>
<td>10⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5 × 10⁻³</td>
<td>47.3 ± 4.5</td>
<td>+20.4% &lt;0.05</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td>55.2 ± 5.2</td>
<td>+40.5% &lt;0.01</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10⁻²</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Macrophages (2.4 × 10⁶/ml) were incubated for 15 min at 37°C in a humidified atmosphere of 5% CO₂ in the presence of different substances: medium alone (control), EDTA 100 mM alone, calcium chloride alone at 5 mM or 10 mM, EDTA 100 mM together with calcium chloride 5 mM, and EDTA 100 mM together with calcium chloride 10 mM. The reaction was stopped and the adherence indices were calculated. Each result is the mean ± SEM of five separate experiments performed in duplicate. Statistical significance was determined by Student’s unpaired t-test.

Discussion

The present study demonstrated that EDTA significantly inhibited the substrate adherence capacity of casein elicited macrophages. The sensitivity of cells to an EDTA concentration as low as 50 mM, lower than concentrations of EDTA used in endodontics, and the potent inhibitory effect of higher EDTA concentrations (100–300 mM), which are similar to that used in root canals preparation (10–15% EDTA = 300–400 mM EDTA) (2, 3, 4), suggest that the EDTA inhibition of macrophage adherence may have physiological significance in vivo at the level of periapical tissues. Moreover, EDTA concentrations ranging from 50 mM to 200 mM can be found in periapical tissues as a result of EDTA leakage during root canals preparation. These correspond to EDTA concentrations that caused half maximal inhibition of AI (IC₅₀): 192 ± 20 mM.

Macrophages play an essential role in the immune response of the host to inflammatory and infectious processes, but the molecular mechanisms involved are poorly defined (12). The phagocytic cell adherence to a smooth plastic surface is comparable to that taking place in animal tissues (16, 17, 18). However, since adherence is the first step in the phagocytic process and essential for macrophage function (13), the inhibitory effect produced by EDTA on adherence suggests that EDTA may inhibit phagocytosis in macrophages.

The fact that the inhibitory effect of EDTA on the AI decreased in the presence of calcium chloride suggests that this effect depends on its chelating action on calcium ions. The stimulatory effect of calcium chloride on AI also supports this suggestion. In this respect, it has been shown that the substrate adherence capacity of monocytes requires the presence of calcium (13).

Although it has been considered that apical extrusion of EDTA during root canal therapy did not affect calcified tissues (2, 3), the inhibitory effect of EDTA on the AI of inflammatory macrophages in this study suggests that when EDTA leakage to periapical tissues occurs, this substance could inhibit phagocytosis and antigen presentation by macrophages, decreasing phagocytosis, and inflammation. However, EDTA has been shown to act as an inflammatory stimulus in dorsal muscle of albino rats (14).

Because of these findings, the use of EDTA in conservative dentistry or in endodontics therapy must take into account that periradicular leakage of this substance not only causes a decalcifying action on periradicular bone but may also have other effects on the immune system.

Many times root canal treatment must be performed in teeth in which pulps are still vital and in which an acute apical periodontitis is present. On other occasions, endodontic treatment is performed in teeth with necrotic pulps and evidence of chronic periodontitis. In both cases it is possible that if we use EDTA for root canal chemomechanical enlargement this substance may leak into periapical tissues through the apical foramen and act on macrophages involved in inflammatory reactions. This action may have either negative or positive consequences. On the one hand, the inhibition of the substrate adherence capacity of inflammatory macrophages may facilitate the inflammatory process resolution contributing to its healing. On the other hand, it could decrease the phagocytic function of macrophages, which is essential for debris removal, retarding the healing of the lesion, or contributing to its chronicity. Moreover, because inflammatory macrophages are implicated in the production of osteoclast activating factor (8), EDTA may even alter bone resorption.

Therefore, we conclude that chemomechanical preparation of root canals using EDTA should be performed carefully to avoid EDTA leakage, because EDTA may reduce macrophage adhesion, thus inhibiting phagocytic function and antigen presentation and modulating the immune response and the inflammatory reactions in periapical tissues.

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References

7. Stern MH, Peizer S, Mackler BF, Levy BM. Isolation and characteriza-
You Might be Interested

Influenza is serious business. Most authorities suggest restricting administration of vaccine to high-risk groups such as the elderly, chronically ill, and health professionals. A recent study, however, showed a reduction of 25% in upper respiratory infections in a group of healthy adults who received vaccine (New Eng J Med 333:889). Side effects must always be considered, of course.

Zachariah Yeomans