In Vitro Inhibitory Effect of EGTA on Macrophage Adhesion: Endodontic Implications

Juan J. Segura-Egea, DDS, MD, PhD, Alicia Jiménez-Rubio, DDS, MD, PhD, José V. Rios-Santos, DDS, MD, PhD, Eugenio Velasco-Ortega, DDS, MD, PhD, and Juan R. Calvo-Gutierrez, MD, PhD

Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) is a specific calcium ion chelator proposed as an endodontic irrigant. This study investigates the effect of EGTA on substrate adherence capacity of rat inflammatory macrophages. 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 was calculated. Results showed that EGTA decreased substrate adherence capacity of inflammatory macrophages in a time- and dose-dependent manner. The EGTA concentration that caused half-maximal inhibition (IC₅₀) was 202 ± 32 mM (p < 0.01). EDTA was more potent than EGTA in inhibiting macrophage adherence (IC₅₀ = 185 ± 22 mM). Calcium chloride (10 mM) decreased the EGTA inhibitory effect on adherence index by 60.2% (p < 0.01). We conclude that EGTA significantly decreased substrate adherence capacity of macrophages.

Conversely, it has been demonstrated that inflamed periapical tissues contain a variety of immunocompetent cells, with macrophages predominating (7). Macrophages play an essential role in periapical lesion development by acting as antigen-presenting cells to memory T lymphocytes (8). This macrophage function requires the adherence to antigen as a first step (9).

The purpose of this study was to evaluate the effect of EGTA on substrate adherence capacity of inflammatory macrophages to determine if EGTA leakage during root canals preparation could modulate macrophage function altering inflammatory reactions in periapical tissues.

MATERIALS AND METHODS

EGTA, 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-h 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.

Collection of inflammatory macrophages was carried out as described previously (10). Mean cells per rat varied from 20 to 30 × 10⁶, 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 94%.

The quantification of substrate adherence capacity was carried out according to the technique described previously by De la Fuente et al. (11) with minor modifications. Aliquots of 180 μl of cell suspension were dispensed in Eppendorf tubes, which resembled the adherence to tissues (11, 12). EGTA (20 μl) was added to a final concentration ranging from 2 mM to 300 mM. Medium (20 μl) was added instead of EGTA 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
The adherence index (AI) was calculated according to the following equation:

\[ AI = 100 - (\text{nonadherent macrophages/ml} / \text{initial macrophages/ml}) \times 100 \]

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

EGTA inhibited substrate adherence capacity of macrophages in all conditions tested. The inhibitory effect of EGTA was a time- and dose-dependent phenomenon (data not shown). The concentration-effect curve for the effects of EGTA on the adherence index of macrophages is shown in Fig. 1. The lowest EGTA concentration that caused a significant inhibition of AI was 50 mM (\(p < 0.05\)) and half maximal inhibition (IC50) was obtained at 202 ± 32 mM (\(p < 0.01\)). EDTA was more potent than EGTA in inhibiting macrophage adherence (IC50 = 185 ± 22 mM) (\(p < 0.01\)).

Incubation of cells with 5 mM of calcium chloride alone increased AI of macrophages by 7.4% (\(p > 0.05\)) (Table 1). Calcium chloride at 10 mM increased AI of macrophages by 13.5% (\(p < 0.05\)). When 100 mM of EGTA was added to the incubation medium together with 5 mM of calcium chloride (CaCl2), the inhibitory effect of EGTA on AI decreased by 34.9% (\(p < 0.05\)).

Calcium chloride at 10 mM decreased the EGTA-effect on AI by 60.2% (\(p < 0.01\)).

### DISCUSSION

This study demonstrates that EGTA significantly inhibited the substrate adherence capacity of inflammatory macrophages. The sensitivity of cells to an EGTA concentration as low as 50 mM, lower than concentration of EGTA proposed by Çalt and Serper (6) as endodontic irrigant (17% EGTA = 400 mM), and the inhibitory effect of higher EGTA concentrations (100–300 mM) suggest that the EGTA inhibition of macrophage adherence could be significant in vivo at the level of periapical tissues if EGTA leaks into periapical tissues.

The fact that the inhibitory effect of EGTA on the AI decreased in the presence of calcium chloride corroborates that this effect depends upon its specific 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 (9).

EGTA is a specific calcium ion chelator. Chelators react with calcium ions in the hydroxyapatite crystals to produce a metallic chelate. Removal of calcium ions from the dentine makes the dentinal tissue softer, especially the hydroxyapatite-rich peritubular dentin and increases the diameter of exposed dentinal tubules (13). EDTA is the chelator that classically has been used in chemomechanical enlargement of canals (13). EDTA chelates with calcium ions and other divalents cations. The combination of NaOCl and EDTA caused a progressive dissolution of the dentin at the expense of peritubular and intertubular areas, so that the diameter of tubular orifices on the instrumented canal walls were enlarged to 2.5 to 4 \(\mu\)m (14). Çalt and Serper (6), investigating the effects of EGTA as an alternative to EDTA in endodontics, found that the combination of NaOCl and EGTA completely removed the smear layer and opened the dentinal tubule orifices. Moreover,

### Table 1. Modulation by calcium chloride of the EGTA effect on substrate adherence capacity of inflammatory macrophages

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (M)</th>
<th>AI Increment (%)</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>72.8 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>CaCl2 5 (\times 10^{-3})</td>
<td></td>
<td>78.2 ± 7.2</td>
<td>+7.4*</td>
</tr>
<tr>
<td>CaCl2 10 (\times 10^{-2})</td>
<td></td>
<td>82.7 ± 7.6</td>
<td>+13.5*</td>
</tr>
<tr>
<td>EGTA 10 (\times 10^{-1})</td>
<td></td>
<td>47.9 ± 4.8</td>
<td>−34.2*</td>
</tr>
<tr>
<td>EGTA 10 (\times 10^{-1}) +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl2 5 (\times 10^{-3})</td>
<td></td>
<td>56.6 ± 5.3</td>
<td>+18.2†</td>
</tr>
<tr>
<td>EGTA 10 (\times 10^{-1}) +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl2 10 (\times 10^{-2})</td>
<td></td>
<td>62.9 ± 6.2</td>
<td>+31.3†</td>
</tr>
</tbody>
</table>

* Compared with control.
† Compared with EGTA 100 mM alone.
these authors observed that EGTA was somewhat effective in removing the smear layer without inducing erosive action, as EDTA had. In accordance with their findings, EGTA could be used as endodontic irrigant but it seems to be less potent than EDTA in removal of smear layer (6).

Substances used in root canal treatment can leak into periapical tissues, altering the function of macrophages and immune cells. In this context, Segura et al. (15) and Jiménez-Rubio et al. (16) demonstrated that several agents used as endodontic irrigants, such as EDTA, hypochlorite, and glutaraldehyde, significantly inhibit macrophage adherence. The inhibitory effect of EGTA on macrophage adhesion showed in this study is in agreement with the previous results of Segura et al. (15), showing the inhibitory effect of EDTA on macrophage adherence to plastic surfaces. However, the potency of EGTA in inhibiting macrophage adhesion is lower than that of EDTA. This could be an advantage of EGTA with respect to EDTA in the case of leakage into periapical tissues.

Timpawat et al. (17) demonstrated that the removal of the smear layer caused significantly more apical microleakage than when the smear layer was left intact. Taking into account that both EDTA and EGTA remove smear layer, the use of these substances as endodontic irrigant increases the possibility of microleakage and the risk of altering macrophage function at the level of periapical tissues.

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 (9). The phagocytic cell adherence to a smooth plastic surface is comparable to that taking place in animal tissues (10–12). Because adherence is the first step in the phagocytic process and essential for macrophage function (9), the inhibitory effect produced by EGTA on macrophage adherence suggests that EGTA inhibits phagocytosis in macrophages.

In the light of these findings, if EGTA is used as irrigant in endodontic therapy, it must be remembered that periapical leakage of this substance not only causes a decalcifying action on periapical bone but may also inhibit phagocytic function and antigen presentation of macrophages, altering the immune response and the inflammatory reactions in periapical tissues.

Drs. Segura-Egea, Rios-Santos, and Calvo-Gutierrez are affiliated with the Department of Stomatology, School of Dentistry, University of Seville. Dr. Jiménez-Rubio is affiliated with the Department of Morphological Sciences, School of Medicine, University of Seville. Dr. Calvo-Gutierrez is affiliated with the Department of Medical Biochemistry and Molecular Biology, School of Medicine, University of Seville, Spain. Address requests for reprints to Dr. Juan J. Segura Egea, C/ Cueva de Menga nº 1, portal 3, 6º-C, 41020 Sevilla, Spain.

References