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## TABLE OF CONTENTS

[\[INTRODUCTION\]](#) [\[MATERIALS AND...\]](#) [\[RESULTS\]](#) [\[DISCUSSION\]](#) [\[CONCLUSIONS\]](#) [\[REFERENCES\]](#) [\[FIGURES\]](#)

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# Epidermal Growth Factor in Liposomes May Enhance Osteoclast Recruitment during Tooth Movement in Rats

Karina Renata Gomes Cimini Saddi;<sup>a</sup> Guilherme Dias Alves;<sup>b</sup> Tony Paiva Paulino;<sup>c</sup> Pietro Ciancaglini;<sup>d</sup> Jose Bento Alves<sup>e</sup>

## ABSTRACT

**Objective:** To evaluate the effects of local administration of epidermal growth factor (EGF) located within liposomes on recruitment of osteoclasts during mechanical force in rats.

**Materials and Methods:** An orthodontic elastic band was inserted between the left upper first and second molars, to move mesially the first molar. Rats were randomly divided into 4 groups (n = 8): EGF (2 ng/μL) located within liposomes (group 1), liposomes only (group 2), soluble EGF (2 ng/μL; group 3), or vehicle alone (group 4). The solutions were injected into the region of the root furcation of the left first molar after elastic band insertion. Tooth movement was measured using a plaster model of the maxilla, and the number of osteoclasts recruited at the pressure side of the first molar was histologically evaluated.

**Results:** Intergroup analysis showed that there was no significant difference between group 2 and group 4 ( $P > .05$ ) and between group 1 and group 3 ( $P > .05$ ). However, group 1 and group 3 exhibited greater differences in tooth movement than group 2 and group 4 ( $P < .05$ ). On the other hand, group 1 showed greater tooth movement than groups 2 and 4 with statistical significance ( $P < .01$ ). The increase in the number of osteoclasts in group 1 was significantly higher than in the other groups ( $P < .05$ ).

**Conclusion:** Exogenous EGF-liposome administration has an additive effect when compared with soluble EGF on the rate of osteoclast recruitment, producing faster bone resorption and tooth movement.

**KEY WORDS:** EGF, Liposome, Osteoclasts, Tooth movement, Orthodontic movement.

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## INTRODUCTION [Return to TOC](#)

Orthodontic procedures are widely used around the world to correct occlusal disturbances and esthetics. However, problems such as the long time that braces must be worn, the pain involved during adjustment, and the visible stigma of braces are still major issues for several patients. Although some improvement has been achieved to ameliorate the visible problem, little has been done to develop new

therapeutic strategies to reduce the length of treatment.<sup>1</sup>

Tooth movement during orthodontic treatment requires remodeling of periodontal tissues, especially in the alveolar bone. Force application disrupts the equilibrium that exists between bone formation and resorption, resulting in more bone resorption than formation on the pressure side and more bone formation than resorption on the tension side.<sup>2</sup> Resorption of bone by osteoclasts is coupled with subsequent bone formation by osteoblasts. Local and systemic factors including cytokines, hormones, growth factors, and mechanical stimulation activate osteoblasts to produce the receptor activator of NF- $\kappa$ B ligand (RANKL), which is vital for osteoclast differentiation and activity. RANKL is expressed on the surface of osteoblastic cells and bone marrow stromal cells and binds to the RANK receptor on the surface of osteoclastic precursors, thereby stimulating the differentiation and activation of osteoclasts.<sup>3</sup>

Local administration of factors that modulate bone remodeling is expected to significantly influence tooth movement. Hormonal and local factors that modulate bone remodeling, such as parathyroid hormone (PTH), 1,25(OH)<sub>2</sub>-D<sub>3</sub>, interleukin (IL) -1, IL-6, and prostaglandin (PGE), significantly influence tooth movement.<sup>4</sup> Recently, the use of epidermal growth factor (EGF) to develop a suitable environment and stimulate selective cell differentiation and proliferation has been proposed. In addition, it has been suggested that it may be involved in the responses of osteoblastic cell lines to mechanical stimuli.<sup>5</sup>

EGF is a small polypeptide growth factor found in a variety of tissues (kidney, submandibular glands) and body fluids (saliva, amniotic fluid) that primarily stimulates epithelial and mesenchymal cell proliferation and differentiation.<sup>6</sup> It also affects other cellular functions such as macromolecule synthesis and bone resorption, and it is considered a natural regulator in the initiation of tooth eruption.<sup>7</sup> It has been shown that EGF is expressed in the dental follicle and alveolar bone before and during prefunctional eruption,<sup>8</sup> suggesting a possible effect on osteoclast recruitment and differentiation. However, the role of EGF on osteoclast behavior during orthodontic tooth movement has not been evaluated so far.

The potential of growth factors as therapeutic agents for the repair and the acceleration of biological processes has been well established experimentally<sup>9</sup>; however, they still face several substantial clinical obstacles, such as the fate of a growth factor administered topically to a wound, which is an enzymatically hostile environment to proteins and polypeptides.<sup>10</sup> The low and short-term bioavailability of the growth factor is not sufficient to make significant therapeutic impacts. Since growth factors are agents that act at specific stages of the cell cycle, their presence at the target area is required in active form and for prolonged periods.<sup>11</sup> Attempts to improve the situation by frequent dosing and/or high doses have not proved useful and can introduce new obstacles to the healing biological action.

One of the persisting key research issues in the design of drug delivery systems is how to achieve significant cytoplasmic delivery of drug molecules. Liposomal formulations have been used to both enhance absorption and regulate release of incorporated drugs, thus localizing the effect of the drugs, both enhancing local and decreasing systemic drug concentrations.<sup>12</sup> It has been shown that liposome encapsulation does not impair the biological activities of polypeptides, such as tumor necrosis factor (TNF)<sup>13</sup> and EGF,<sup>14</sup> whereas it protects them from proteases.<sup>15</sup>

Thus, the aim of the present work was to investigate the effects of exogenous EGF administration located within liposomes on osteoclast recruitment and tooth movement after mechanical force application.

## MATERIALS AND METHODS [Return to TOC](#)

Male Holtzman rats weighing 250 to 350 g were included in the study. The animals were kept in plastic cages with access to food and water ad libitum. Prior to the surgical procedures, all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the Animal Committee of the University of Uberaba.

All solutions were prepared by using Millipore DirectQ ultra pure apyrogenic water.

Dipalmitoylphosphatidylcholine (DPPC), lysophosphatidylcholine (LPC), ethylenediaminetetraacetic acid (EDTA), formaldehyde, ethanol, chloroform, and glycerol were purchased from Sigma Chemical Co (St Louis, MO). The analytical grade reagents were used without further purification.

### EGF-Liposome Preparation

EGF was dissolved in 1 mL of 10 mM acetic acid containing 0.1% (w/v) bovine serum albumin in a final concentration of 0.2  $\mu$ g/mL and stored at -20°C. EGF stock solution was then diluted to 2 ng/ $\mu$ L in phosphate-buffered saline (PBS) previously sterilized in a 0.2- $\mu$ m filter.

The liposome was obtained (by sonication) using 3.0 mg/mL DPPC, 0.3 mg/mL LPC, and 10  $\mu$ L EGF solution, producing homogeneous unilamellar vesicles containing 0.5 ng/ $\mu$ L of EGF with a ~ 100-nm diameter.

### Tooth Movement in Rats

The animals were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally). Experimental tooth movement was achieved by interproximal insertion of an elastic band between the left upper first (M1) and second molars (M2), according to the method described by Waldo and Rothblatt.<sup>16</sup> The right upper first molars served as control, without elastic band insertion or drug administration.

The rats were assigned to one of the following groups:

group 1 (GI; n = 8), administration of 20 ng EGF-liposomes in 10  $\mu$ L PBS solution;

group 2 (GII; n = 8), administration of liposomes in 10  $\mu$ L PBS solution;

group 3 (GIII; n = 8), administration 20 ng EGF in 10  $\mu$ L PBS solution; and

group 4 (GIV; n = 8), administration of 10  $\mu$ L PBS solution.

Each solution was injected in the mucosa adjacent to the elastic band insertion using a microsyringe (Hamilton, Bonaduz, Switzerland) just after the elastic band insertion.

After 5 days of elastic band insertion, rats were anesthetized with ketamine (90 mg/kg) and xilazine (5 mg/kg), and after decapitation, the maxillae were retrieved for the following analysis.

### Measurement of Tooth Movement

After elastic band removal, a precise plaster model of the maxilla was prepared by an impression with silicone material (silon) and dental stone. The distance between the first and second molars was measured in plaster replicas of the experimental and control sides with dial calipers (Digimesse®, São Paulo, Brazil) with a minimal graduation of 0.01 mm. Experimental tooth movement was calculated as the difference between distances at the experimental and the control sides.


### Histological Analysis

After the plaster replica, the specimens were fixed in 4% neutral formalin for 48 hours and subsequently demineralized in a 10% EDTA solution (pH 7.2) for 6 to 8 weeks. The samples were dehydrated in ascending concentrations of ethanol and embedded in paraffin wax. Longitudinal, serial, 6- $\mu$ m-thick sections of the molars were stained with hematoxylin and eosin and examined under a light microscope (Olympus BX 50).

### Determination of Osteoclast Number

To determine the number of osteoclasts, 12 randomly chosen sections were averaged for both the experimental and control sides. The osteoclasts were defined as multinuclear cells on the bone resorption lacunae.<sup>17</sup>


The slide images were obtained at a final magnification of 400 $\times$  using a capture plate and microscope (Olympus BX 50) interfaced with a personal computer and software image tool.

The osteoclast number was counted using a grid with vertical and horizontal lines placed on the photomicrograph, with the aid of the Confocal Assistant (Chapel Hill, NC). All osteoclasts located on the grid line intersections were counted ([Figure 1](#) ) by a person who was previously calibrated. The osteoclast number were carried out at double blind fashion.

### Statistical Analysis

The difference between the groups regarding the evaluated parameters was tested by using an analysis of variance (ANOVA) followed by the Tukey test. The data of all groups passed the Kolmogorov-Smirnov test of normality. All groups were carried out in triplicate and significance was accepted when  $P \leq .05$ .

## RESULTS [Return to TOC](#)

[Figure 2](#)  shows the amount of tooth movement. Intergroup analysis showed that there was no significant difference between GII and GIV (0.26 and 0.26 mm, respectively,  $P < .05$ ) and between GI and GIII (0.55 and 0.42 mm, respectively,  $P < .05$ ). However, GI and GIII exhibited greater differences in tooth movement than GII and GIV ( $P < .05$ ).

To determine whether the additive effect of exogenous EGF on tooth movement was related to the enhancement of osteoclast

recruitment and/or differentiation on the alveolar bone surface, we evaluated the number of osteoclasts by counting the multinuclear cells stained with hematoxylin and eosin. As shown in [Figure 3](#), the test side of all groups exhibited a higher number of osteoclasts on the alveolar bone surface when compared with the control side ( $P < .05$ ). The increase in the number of osteoclasts in GI was significantly higher than in the other groups ( $P < .05$ ).

## DISCUSSION [Return to TOC](#)

Orthodontic tooth movement depends on the ability of periodontal cells to react to mechanical stimuli. The most prominent features are the remodeling of the periodontal ligament and the resorption of alveolar bone. The rate at which a tooth can move through the bone is supposed to be restricted primarily by the number and activity of the osteoclasts.<sup>18</sup>

Several experimental approaches have been attempted to induce more efficient tooth movement by increasing the number of osteoclasts and the resultant bone resorption. Simultaneous administration of  $1,25(\text{OH})_2\text{D}_3$ ,<sup>17</sup> osteocalcin,<sup>19</sup> PGE,<sup>20</sup> vascular endothelial growth factor,<sup>21</sup> and application of mechanical force induces the differentiation of osteoclasts, enhancing bone resorption and resulting in faster tooth movement than those produced by mechanical force alone. Furthermore, it has been observed that bone resorption in response to IL-1 or TNF is accompanied by an increase in osteoclast number.<sup>22</sup>

Soluble factors such as IL-6, PTH, PGE, TNF $\alpha$ , EGF, IL-1, and platelet-derived growth factor may influence osteoclast and osteoblast function. These factors may enhance the activity of the osteoclast either indirectly, by stimulating the expression of RANKL on osteoblasts, or through direct effects on osteoclast and osteoblast function.<sup>23</sup> However, because these drugs are rapidly flushed by blood circulation, daily systemic administration or daily injection is needed, requiring several doses per day, interfering in the animal's health.

In the present study, we investigated whether local administration of EGF would accelerate osteoclast differentiation after mechanical force application. Experimental tooth movement was achieved by interproximal insertion of an elastic band between the left upper first and second molars. The use of elastic bands is not indicated to provide a controlled force delivery system for experimental tooth movement in rats.<sup>24</sup> However, this device produces a momentary force on periodontal tissue and alveolar bone for 3 days<sup>25</sup> and has been used by several authors in association with drug administration<sup>26–28</sup> or to investigate the mechanisms of bone formation during tooth movement by in situ hybridization.<sup>29</sup>

Liposome-encapsulated drugs have been widely used in the past few years, achieving slower release and higher penetrative capacity through tegumental barriers.<sup>30</sup> The encapsulated drugs continuously diffuse into the surrounding tissues and may reside at a considerable concentration around the injected area, enhancing the effects of their pharmacological agents for a longer period. Growth factors are agents that act at specific stages of the cell cycle, which require their presence for prolonged periods. We used liposomes to carry EGF in an attempt to minimize traumatism and stress and to increase the time of permanence of EGF at the application site. In our experiments, the use of EGF encapsulated in liposomes increased the number of osteoclasts and enhanced tooth movement when compared with soluble EGF and hollow liposome. We may suggest that soluble EGF was promptly metabolized while EGF in liposomes could act for a longer time. Oral administration of exogenous EGF has been reported to have no effect on alveolar bone remodeling during molar drift and orthodontic tooth movement in sialoadenectomized rats.<sup>31</sup>

Because the number of osteoclasts increased at the experimental side of EGF-liposome administration, we suggest that EGF increased the recruitment of osteoclasts and/or promoted its differentiation. In fact, a number of cytokines and growth factors have been implicated in the regulation of bone resorption. Colony-stimulating factor-1 (CSF-1) has been shown to be a prerequisite for osteoclast formation and for proliferation, differentiation, activation, and survival of macrophages.<sup>32</sup> Furthermore, transcription factors including *c-fos* and NF- $\kappa$ B play an essential role in stimulating the differentiation of the committed precursors to mature osteoclasts. It has been shown that transcription factors (*c-fos* and NF- $\kappa$ B) and the osteoclast differentiation factor may be required for osteoclast formation. Gene expression of *c-fos* is enhanced by CSF-1 or EGF,<sup>33</sup> and NF- $\kappa$ B appears to be stimulated only by IL-1 $\alpha$ .<sup>34</sup> Further studies should be designed to determine the real potential of EGF and liposomes on bone metabolism and the factors involved.

## CONCLUSIONS [Return to TOC](#)

- Local administration of EGF encapsulated in liposomes, associated with a mechanical force, induced greater osteoclast recruitment when compared to other groups.
- Local injections of EGF-liposome could be used and are probably applicable to orthodontic therapy in order to enhance bone resorption.

## ACKNOWLEDGMENTS

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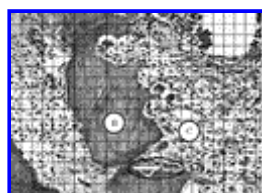
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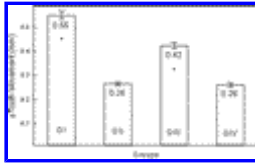
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**FIGURES** [Return to TOC](#)



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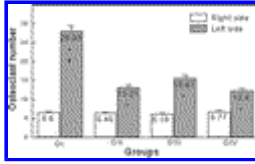
**Figure 1.** Histological illustration of periodontal healing 5 days after elastic band insertion (hematoxylin and eosin staining). Determination of the number of the osteoclasts on the alveolar bone surface on the pressure side was made using 12 sections selected at 5 intervals and averaged for each animal: (arrow) osteoclast, (B) bone, (C) periodontal ligament (original magnification 400x)



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**Figure 2.** Mean and standard deviation of tooth movement (difference between right and left side) determined after 5 days of treatments.

\*Statistically different,  $P < .05$



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**Figure 3.** Mean and standard deviation of the number of osteoclasts determined after 5 days of treatment for all experimental groups.

\*intragroup analysis; #intergroup analysis; statistically different,  $P < .05$

<sup>a</sup> MS student, Department of Orthodontics, Pontificia Universidade Católica de Minas Gerais, Belo Horizonte, MG, Brazil

<sup>b</sup> Graduate student, Department of Basic Sciences, Universidade de Uberaba, Uberaba, MG, Brazil

<sup>c</sup> Associate Professor, Department of Basic Sciences, Universidade de Uberaba, Uberaba, MG, Brazil

<sup>d</sup> Associate Professor, Department of Chemistry, Faculdade de Filosofia Ciências e Letras, Universidade de São Paulo, Ribeirão Preto, Brazil

Corresponding author: José Bento Alves, PhD, Universidade de Uberaba, Av. Nenê Sabino, 1801. Bairro Universitário, 38.055.500. Uberaba, MG, Brazil (E-mail: [propg.pr@uniube.br](mailto:propg.pr@uniube.br))