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## Effect of root perforation repair materials on morphology and attachment behavior of human PDL fibroblasts *in vitro*

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تأثير المواد المرممة للثقيب الجذري على شكل وإرتباط الخلايا الصانعة للليف (دراسة مخبرية) هدفت هذه الدراسة المخبرية باستعمال المجهر الإلكتروني إلى تقييم شكل وإرتباط الخلايا الصانعة للليف إلى المواد التي تستخدم كحشوات لإصلاح ثقب الجذور، هذه المواد هي (كمبوزيت سبكرم في بي اتش، المنيرال ترائي أو أكسيد(إم تي آيه)، والغوتابر شا المذابة بالحرارة) فوجي - تو - ل - سي - جلاس أيونمر، شملت الدراسة على أربعة ومائتين شريحة جذرية من أسنان بشرية مخلوغة. وقد جرى تحضير تجويف في منتصف كل شريحة جذرية بمثقّب دائري رقم ٢، وتم وضعت مواد الاختبار في الفجوات المعدة في منتصف كل شريحة. ثم وضعت شرائح الجذور في أحواض إستنبات نسيجية، ووضع فوق كل شريحة ١ مل من خلايا الرباط حول السنّي الصانع للليف. أما بالنسبة لمجموعة شرائح المراقبة الإيجابية فقد وضع ٥ و ١٠ مل من المتماز على معلق الخلايا الذي أضيف لها في حوض الإستنبات. وبعد ذلك جرى حضن الخلايا لمدة ٤ ساعات، و ٢٤ ساعة، وإسبوع واحد تحت ٣٧ مئوية و ١٠٠% رطوبة. أظهرت النتائج وجود خلايا كروية، ومتعرجة ولها نتوءات مع كل من مجموعة المراقبة الإيجابية، مجموعة الراتنج المركب، ومجموعة إم تي آيه الغير جافة. مجموعة إم تي آيه الجافة ومجموعة الغوتابر شا أظهرت كمية كبيرة من خلايا دائرية ومسطحة قليلاً مع سطح أملس. مجموعة الامتصاص الزحاجي الشاردي أظهرت أقل عدد من الخلايا ولكن أفضل إنتشار وإنبساط للخلايا على سطحها. يستنتج مما سبق أن إرتباط خلايا الرباط السنّي الصانع للليف يعتمد على مدى التوافق الحيوي للمادة المستخدمة.

The morphology and attachment behavior of human periodontal ligament fibroblasts (HPDLF) to different root perforation repair materials of TPH spectrum composite, mineral trioxide aggregate (MTA), thermoplasticized gutta-percha (GP) and Fuji II LC glass-ionomer were evaluated using a scanning electron microscope (SEM). A total of 84 root slices of human teeth were used. A perforation was done in the center of each root slice with #2 round diamond bur and sealed with one of the tested materials. The experiments were performed in tissue-culture 24-well plates in which 1 ml of human periodontal ligament fibroblast cell suspension was placed over the filling materials and the control root slices. For the positive-control group, 0.5 ml of monomer was added to the cell suspensions before being dispensed into the wells. The cells were incubated for 4 hours, 24 hours and one week at 37°C and 100% humidity. Results showed round cells with less smooth surfaces and many rough blebs in the positive control, composite resin and with the freshly prepared MTA groups. The set MTA and GP groups showed large amount of round and slightly flattened cells with smooth surfaces. The glass-ionomer groups showed the least number of cells and the best spread of the cells on the material surface. It was concluded that the cell attachment depend on the biocompatibility of the material used.

### Introduction

Root perforation is a communication between the root canal system and periodontal ligament through the floor of the pulp chamber or root canal wall. It can occur as a result of carious lesion, internal or external root resorption during endodontic instrumentation and post space preparation. The prognosis of an endodontic perforation depends on the size and location of the defect, and the rapidity of sealing the perforation area with biocompatible material. A wide variety of materials such as zinc oxide eugenol, amalgam, Cavit, composite resin, glass-ionomer and Mineral Trioxide Aggregate (MTA) have been suggested to seal these perforations.<sup>1-4</sup>

*In vitro* assays for cytotoxic effects are increasingly being used for an initial screening of dental materials intended for use in humans. Thus, it is possible to study the cytotoxicity of perforation repair materials with reliability and reproducibility

using the most appropriate normal human cells such as the human PDL fibroblasts (HPDLF).<sup>3, 4</sup>

Cells derived from the periodontal ligament (PDL) are responsible for normal maintenance and regeneration of the periodontium.<sup>5</sup> The ultimate goal of treatment of root perforations is to maintain or reestablish the damaged attachment apparatus. To achieve this, the selected material has to be biocompatible with the PDL cells. Therefore, the aim of this study was to examine, *in vitro*, the morphology and the attachment behavior of the HPDLF to different perforation repaired materials of composite resin, MTA, thermoplasticized gutta-percha and resin modified glass-ionomer (RMGI) using scanning electron microscope.

### Materials and Methods

#### Test Materials

Eighty-four human root slices were divided into four experimental groups of 18 root slices each and 12 for positive and negative control. The root perforations filling materials used were:

1. TPH Spectrum composite (Dentsply Caulk, Milford, USA #542413) for group 1.
2. MTA (Dentsply, Tulsa Dental Products, Tulsa, OK, USA #5415547) for group 2.

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3. Thermoplasticized gutta-percha (Obtura, Obtura Co., USA) for group 3.
4. Light-cured glass-ionomer (Fuji II LC, GC Corporation, Tokyo, Japan #261121) for group 4.

### Culture of the Human Periodontal Ligament Fibroblasts (HPLF)

These were obtained from previously established stocks that had been stored in liquid nitrogen.<sup>6</sup> The HPLF used in this study were between their fourth and fifth passage in culture. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with 2mM L-glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin.

### Preparation of the Specimens

Forty-two single roots of freshly extracted human teeth with completely formed roots and no resorption, or cracks were collected. The selected teeth were cleaned of any attached bone or soft tissue tags and stored in 0.9% normal saline solution until used. The crowns of the teeth were removed at the cemento-enamel junction using a water-cooled 170 L carbide bur attached to a high-speed handpiece. The roots were sectioned longitudinally in buccolingual plane with diamond disc operating in a low speed of 60 rpm with water cooling. A 14 mm root slice was made from each half. A total of 84 root slices were collected. A perforation was made in the center of each root slice with water-cooled #2 round diamond bur attached to a high-speed handpiece. All root slices used for the experiment were washed then steam autoclaved at 250°F for 15 minutes. After sterilization, all further procedures were carried out under aseptic condition using sterile instruments. The root slices were washed with saline before and after insertion of the tested materials to mimic the clinical situation. The tested materials were mixed and condensed in the cavities according to manufacturer's instructions and evaluated while they were fresh. Thermoplasticized gutta-percha using Obtura machine was condensed in the perforated cavity after being dried with paper points. AH26 silver free sealer cement\* was used with gutta-percha.

### Experimental Procedures

The growth of the HPDL fibroblasts was examined with a light microscope before starting the experiment. The cells were harvested with 0.02% trypsin in phosphate-buffered saline then washed three times in PBS solution before being suspended in the culture medium. The experiments were performed in a tissue-culture cluster\*\* containing 24 wells, each with an inner diameter of 16 mm. One root slice was placed in each well with the convex surface (cementum) of the root up. One milliliter of cell suspension was added carefully over the filling material then the root slice was totally covered by the cell suspension. For each experimental material, 6 root slices were used per observation periods (4 hours, 24 hours, one week). Twelve root slices without filling materials were used as control where two root slices were used per observation period. For the positive control group 0.5 ml of the monomer was added to the cell suspensions before being dispensed into the wells.

The tissue culture clusters were placed into an incubator at 37°C and 100% humidity. The specimens were incubated for 4 hours, 24 hours, and one week. The medium was not replaced with fresh one during the observation periods. At the end of incubation period, the specimens were prefixed with a few drops of 0.1% glutaraldehyde for 5 minutes. The medium was decanted, and the cells were fixed with 2% glutaraldehyde in 100 mmol/L cacodylate buffer (pH = 7.2) for 30 minutes at room temperature. The specimens were washed briefly with PBS and dehydrated with sequential washes in a series of 50%, 70%, 90%, and 95% ethyl alcohol and twice in absolute ethyl alcohol each time for 30 minutes. The specimens were then critically point-dried with CO<sub>2</sub>\*\*\*, mounted on copper stubs, and coated with gold. The specimens were examined and photographed by using a scanning electron microscope† at an accelerating voltage of 25 kV.

### Results

#### Control

The negative control cultures demonstrated a large number of fibroblasts with normal morphology. They adhered to the cementum surface with microvilli and filopodia (Fig.1). As time progressed, they appeared to be fully spread and

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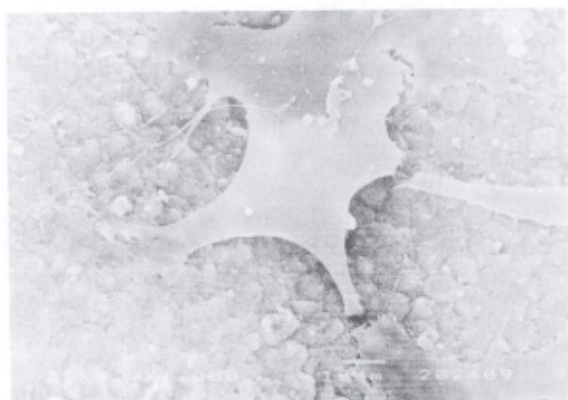
\*\* Linbro, Flow Laboratories, McLean, VA, USA

\*\*\* Samdri-PVT-3B, Tousimis Research Corp, Rockville, MD, USA

† Jeol, JSM, T330A, Electron Optical Laboratory, Tokyo, Japan



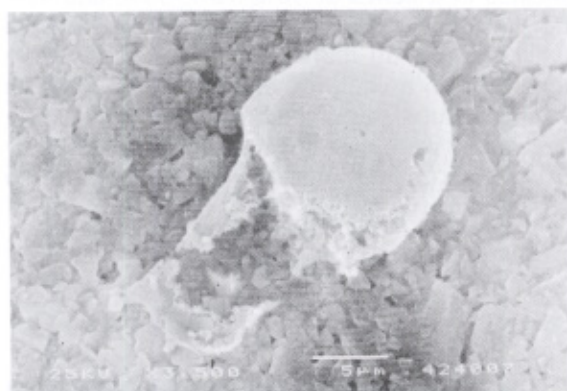
well attached to the cementum by means of lamellipodia. The positive control showed few round cells with many blebs and rough surface.



**Fig. 1.** Scanning electron micrograph of HPDLF (negative control) attached to cementum surface at 24 hours incubation. The spread cell had smooth surface and attached with filopodia and lamellipodia (Original magnification 1000x).

### Composite Resin Material

The composite resin showed good adaptation to the cementum surface and its surface texture was smooth compared to the other tested materials. At the 4 hour-incubation, the fibroblasts were round to discoid in shape, had small vacuoles in the surface and slight attachment to the cementum by means of microvilli and filopodia. As the contact time extended, many vacuoles and blebs appeared on the cell surface with no attachment to the substratum (Fig. 2).



**Fig. 2.** Scanning electron micrograph of HPDLF grown on composite resin at 24 hours incubation. The cell was round in shape with rough surface and many vacuoles (Original magnification 3500x).

### MTA Material

The surface texture of the MTA was slightly rough compared to composite resin, but was adapted well to the cementum surface. At 4 hour-incubation, more cells were seen on the MTA surface. These cells were round in shape and slightly attached to the MTA by means of microvilli and filopodia. Some of these cells have smooth surface and others have vacuoles. As time progressed, the cells increased in number, showed less vacuoles on their surface and some became attached to the MTA by means of lamellipodia. Few damaged cells were seen too.

### Gutta-percha Material

The surface texture of GP was rough and irregular. The material was not well adapted to the cementum surface and large gap between GP material and cementum surface was seen. During the three observation periods, large number of fibroblasts was seen on GP. The cells were round to discoid in shape, have smooth surface, and slightly attached to the GP. Damaged cells were seen rarely. As the incubation period extended, the fibroblasts increased in number and became slightly attached with filopodia. The majority of the cells on the cementum was discoid in shape, have smooth surface and attached well to the surface.

### RMGI Material

The RMGI material showed rough surface and few cells attached to it. The adaptation of the material to the cementum surface was similar to both MTA and composite resin. At 4 hour-incubation, the fibroblasts appeared to be spread and firmly attached by means of filopodia and lamellipodia (Fig.3). Damaged cells were seen rarely. As the incubation period extended, the fibroblasts appeared to be similar to that seen with negative control. Fried egg appearance of fibroblasts was observed on the cementum surface in close proximity to the glass-ionomer material.

### Discussion

The choice of material to repair root perforation is an important factor for good prognosis because perforation repair is usually affected by the material's biocompatibility.<sup>7</sup> In this *in vitro* study, the 4<sup>th</sup> passage of the cultured cells were successfully grown on the tissue culture. The use



of the 4<sup>th</sup> to 5<sup>th</sup> passages of cultured cells was reported by several authors.<sup>6,8</sup> This usually gives a clean, tissue-fragments free culture.

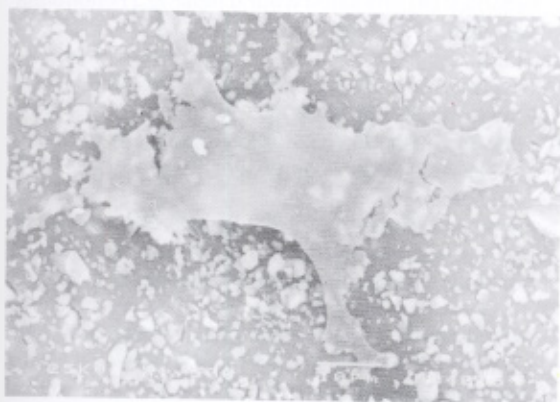


Fig. 3. Scanning electron micrograph of HPDL fibroblasts attached to RMGI at 4 hours. The cell has smooth surface, spread completely and attached with lamellipodia (Original magnification 1500x).

A primary advantage of *in vitro* models is the ability to isolate and study the cellular events apart from the complex interactions of the cells and perforation repair materials that occur during periodontal regeneration procedure *in vivo*. Moreover, tested materials directly contacting fibroblasts could mimic the clinical condition. In such a situation, the actual cytotoxic effects of these perforation repair materials may be seen. A disadvantage of commonly used *in vitro* biocompatibility testing system was the fact that in such assays, only the material's cytotoxicity was studied. Other factors such as the material's physical structure and surface characteristics, known to influence the tissue response to the material, are rarely considered.<sup>5</sup>

The results of the present investigation showed that the surface texture and the composition of the tested root perforation repair materials had an influence on the quantity and quality of the PDL fibroblasts. This is in agreement with the observation of Balto and Al-Nazhan.<sup>6</sup> Adhesion and spreading of the cells on a material surface are the initial phase of cellular function. Zhu *et al.*<sup>9</sup> suggested that cell adhesion and spreading on filling materials could be used as a criterion for evaluation of the biocompatibility of that material. Scanning electron micrograph of the negative control showed that as time progressed, the PDL fibroblasts moved from the stage of rounded with microvilli growth to radial growth of filopodia to the flattening of the cell mass.

Composite resin showed round cells with little or no spreading through the three observation periods. This suggested that the material might be toxic. Resin has been reported to liberate unbound substances from polymerization.<sup>10</sup> It contains methacrylate derivative related to Bis-GMA which has been shown to be not only a cytotoxic agent,<sup>11</sup> but also a genotoxic agent.<sup>12</sup> The cytotoxicity of composite resin was also reported in other studies when tested as root-end filling or as perforation repair material.<sup>1,9,13</sup>

Fresh samples of MTA showed changes in the cell morphology at 4 hours, in which cells appeared round, almost detached from the substrate, and had vacuoles in their surface. The persistence of rounded cells with little or no spreading could be caused by the presence of leachable and toxic components which affect both the morphology and the attachment behavior of the cells. Vacuolization of the cytoplasm is a common finding in injured cells and could be caused by the uptake and storage of early toxic products by the fibroblasts. The presence of the blebs on the surfaces of some injured cells may be due to cytoplasmic shrinkage resulting from toxicity of the test material.<sup>14,15</sup> The set MTA samples were less toxic than fresh samples. Similar results were reported by Balto<sup>14</sup> and Keiser *et al.*<sup>16</sup>

In this study, some of the PDL fibroblasts attached to the gutta-percha by means of filopodia but the majority of the cells were round or discoid with smooth surface. Geurtsen *et al.*<sup>17</sup> tested the cytocompatibility of the gutta-percha with four endodontic sealers and they found that gutta-percha and apexit did not induce PDL cellular alterations, whereas gutta-percha with seal apex or AH26 caused moderate cellular damage. The few damaged cells seen in gutta-percha groups in this study could be due to unset AH26 sealer cement.<sup>17,18</sup>

During the three observation periods, few cells were seen to be attached to the RMGI material. This could be due to the rough surface of the material. However, the cells showed normal morphology similar to that in the negative control. This could be due to the biocompatibility of the material. Tai and Chang<sup>1</sup> found that RMGI and amalgam were less cytotoxic to HPLF than composite resin. Moreover, Makkaway *et al.*<sup>19</sup> reported that the cytotoxicity of amalgam is more than that of RMGI as determined by mitochondria enzyme activity of PDL cells. Drago<sup>20</sup> used RMGI to repair subgingival root perforation caused by caries and he found



clinical and histological evidence of epithelial and connective tissue adherence to the resin-ionomer material. He referred this biocompatibility to the antimicrobial activity of the fluoride release of these materials that affects the composition of the bacteria by altering carbohydrate metabolism.<sup>21,22</sup> In addition, the biocompatibility of glass-ionomer could be attributed to their negligible exothermic reaction on setting and the rapid rise in pH as the material hardens.<sup>23</sup>

In conclusion this study supports the view that cell attachment depends on the biocompatibility of the material used. Both MTA and RMGI showed good adaptation to root surface and provided favorable substrate for HPLF attachment. On the other hand, gutta-percha showed poor adaptation to root surface, but appeared to be suitable substrate for HPLF attachment. Composite resin had good adaptation to the cementum surface but it was the most toxic material used.

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