Evaluation des propriétés biologiques et de scellement de 4 matériaux endodontiques

Galgano, Camillo Michaele

UNIVERSITE DE GENEVE

FACULTE DE MEDECINE
Section de Médecine dentaire
Département de Thérapeutique buccale et d'Orthodontie

Thèse préparée sous la direction du Professeur Ivo KREJCI et du
Docteur Serge BOUILLAGUET, Privat-docent

EVALUATION DES PROPRIETES BIOLOGIQUES ET DE SCELLEMENT DE 4 MATERIAUX ENDODONTIQUES

Thèse

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Camillo GALGANO

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IN VITRO EVALUATION OF THE BIOLOGICAL AND SEALING PROPERTIES OF FOUR ENDODONTIC SEALERS

ABSTRACT

The objectives of this study were to evaluate the biological and sealing properties of 4 endodontic sealers (PCS/ Kerr, RoekoSeal/ Roeko, TopSeal/ Dentsply, EndoREZ/ Ultradent). For cytotoxicity testing, the materials were either placed immediately in contact with cultured cells (Fresh) or 24h after setting (Set). The samples were initially placed in contact with the cells for 24h, then transferred to new cells for another 24 h, and transferred again after 1 week of storage. Cytotoxicities of the materials to cultured fibroblasts were measured by the MTT assay. For the leakage study, twenty extracted human roots were obturated with acrylic cones and sealers and immersed for 48 h into TRICT-labeled lipopolysaccharide. The roots were then sectioned into 0.5 mm thick slabs and observed under a confocal laser scanning microscope to detect the presence of the TRICT-LPS inside the canal. The intensity of LPS- leakage was estimated semi-quantitatively. The results showed that cytotoxicity generally increased with time and that most materials pose significant biological risks, particularly in the freshly mixed condition. Further, all materials showed significant leakage although there was large variation among teeth. Overall, the Roeko Seal was less cytotoxic and more effective in sealing root canals against LPS leakage compared to other materials.

INTRODUCTION

Factors which contribute to the clinical success of endodontic therapy are the complete sealing of the root canal system after cleaning and shaping and the absence of any adverse interaction between the sealer and peri-apical tissues (SCHILDER 1967, SAUNDERS & SAUNDERS 1994). Because endodontic sealers can be extruded through the apex of the root during placement, they should exhibit good biological properties.

Among the most commonly used endodontic sealers are the ZnO-eugenol based, the $Ca(OH)_2$-based, the GI-based and the resin-based root canal sealers. Research has shown that most endodontic sealers are irritants to the living cells (HUANG et al. 2002). ZnO-based endodontic sealers have been used for many years but research has shown that they can release eugenol, which is known to be cytotoxic in vitro (HUME 1986, SCHMALZ et al. 2000). Although $Ca(OH)_2$-based sealers have been shown to exhibit interesting biological properties, the major problem with this type of sealers is that they tend to dissolve over the years (HOVLAND & DUMSHA 1985). For glass-ionomer based endodontic sealers, reports have shown that Ketac Endo can stimulate PGE2 release from cultured human fibroblasts (WILLWESHAUSEN et al. 2000). Resin-based root canal cements are increasingly gaining popularity because they exhibit long-term dimensional stability and do not contain eugenol. However the toxicity of resins and specifically the toxicity and the mutagenicity of resin-based endodontic sealers are well documented (SCHWEIKL et al. 1998, HUANG et al. 2002). Therefore their use remains controversial. More recently, silicon-based materials

have been developed as root canal sealers and preliminary clinical data are promizing (HUUMONEN et al. 2003, WU et al. 2002). However, little is known about their biological properties.

Endodontic sealers are generally associated with core materials like gutta-percha to seal the root canal system and to prevent bacterial infection or re-infection of the peri-apex. Nevertheless many reports have confirmed that a complete sealing of the root canal system is not always achieved with current materials and obturation techniques. This lack of sealing has been demonstrated using dye, fluids, bacteria and radioisotopes as tracers in microleakage studies (WU et al. 1993, CANALDA-SAHLI et al. 1992). However, a majority of the tracers used for such studies are quite different in size and shape from bacteria and endotoxins that might cause problems clinically (CHAVEZ DE PAZ et al. 2003, HILTON 2002). Thus, the relevance of these microleakage studies to clinical problems remains controversial (WU & WESSELINK 1993). BARTHEL et al. (1999) have compared in vitro bacterial and dye leakage tests to measure the seal of root canal fillings and have demonstrated the lack of correlation between the 2 methods. Fortunately, tracer studies have undergone significant evolution in the past decade. COLEMAN (1995) have used LPS to evaluate the leakage beneath cemented crowns. They demonstrated that LPS leakage can occur after 2 weeks despite the sensitivity of the detection method was poor because they used fluorescent microscopy. LAGHIOS et al. (2003) have used LPS to evaluate the sealing of endodontic materials. They reported that teeth with gutta-percha root-end fills can permit leakage of LPS as evidenced by SDS page analysis. Because LPS is a major virulence factor of gram-negative

bacterial pathogens, which are known to activate inflammatory and humoral immune responses their penetration inside the root canal should be avoided (STASHENKO et al. 1998). Confocal microscopy is a non-destructive technique that enables high-resolution images to be made from the sub-surface of the samples and has the potential to pick-up fluorescence from deep within tissues (WATSON et al. 2000). Since LPS can be labeled with fluorescent dyes and observed using confocal laser scanning microscopy their use as a tracer for microleakage studies is promising.

The objectives of this study were (1) to evaluate the cytotoxicity of 4 endodontic sealers to cultured fibroblasts and (2) to compare the sealing characteristics of these sealers using confocal laser scanning microscopy and labeled LPS as tracers. The null hypothesis tested in the current study was that there are no differences between the 4 endodontic sealers in terms of cytotoxicity or sealing ability.

MATERIALS AND METHODS

The four endodontic sealers evaluated in this study were: Pulp Canal Sealer (Batch number: 9-1067; Kerr Hawe SA, Bioggio, Switzerland), RSA Roeko Seal Automix (Batch number: 2110841; Roeko, Langenau, Germany), Top Seal (Batch number: 0111001685, Dentsply De Trey, Konstanz, Germany) and Endo REZ (Batch number: 4D89; Ultradent Products Inc., South Jordan, Utah, USA).

Cytotoxicity testing

Materials were prepared according to manufacturer's instructions, under aseptic conditions to prevent the risk of biological contamination during the cytotoxicity testing (WATAHA et al., 1999). Briefly, the materials were packed into a Teflon mold (1 mm thick x 10 mm diameter) and covered on both sides with Mylar sheets. Thus the ratio of the surface area of the discs to the volume of cell culture medium was 1.88 cm^2/mL as recommended by the International Standards Organization. Samples were placed in contact with cell monolayers immediately after setting (Fresh conditions) to provide conditions relevant to those seen *in vivo* by periodontal ligament cells. A second set of test samples was prepared as previously described and stored at 37°C for 24h before being exposed to the cultured cells. This condition (Set) is relevant to the clinical situation observed after setting.

For cytotoxicity testing, the materials were applied in direct contact with Balb/c 3T3 mouse fibroblasts (ATCC CCL 163, clone A31, American Type Culture Collection, Rockville, MD, USA). Cells were maintained in DMEM with 5 % fetal calf serum, 25 mmol/L of HEPES buffer, 1 µg/mL of gentamycin, 125 units/mL of penicillin, 125 µg/mL of streptomycin, and 2 mmol/L glutamine (all from Oxoid, Basel, Switzerland). Twenty-four hours prior to the addition of the materials, the cells were plated at 25,000 cell/cm^2 in 24-well format (Costar, Cambridge, MA, USA) in 1 mL of medium per well. The cells were incubated at 37° C in 5 % CO_2/95 % air to allow attachment of the fibroblasts to the bottom of the wells.

Cytotoxicity was assessed at multiples times to determine if trends in the biological response were observable. This strategy has been used

successfully in the past with alloys and resins. Cytotoxicity was initially assessed for 24 h. After this interval, the specimens were removed from the cell-culture, rinsed twice with sterile PBS and then immediately added to a second cell-culture (which had been plated with cells 24 h earlier). This second culture was incubated for 24 h and the SDH activity assessed after incubation (48 h reading). The specimens were then retrieved again, rinsed again, and incubated in cell-culture medium with no cells for 5 days at 37°C and 5 % CO_2. Finally, the specimens were rinsed again with PBS and added to a third cell-culture that was plated with cells 24 h before. The SDH activity was then assessed 24 h later (1 week reading).

For each of the three time points (24 h, 48 h, or 1 week), the cells were incubated for 24 h in presence of the materials or Teflon discs (negative controls) before assessing cellular activity by measuring mitochondrial succinic dehydrogenase (SDH) activity. SDH activity was measured by means of the MTT colorimetric assay (WATAHA et al. 1992). SDH activity was quantified by dissolving the MTT-formazan into 0.1 N NaOH (6.25 %) in dimethyl sulfoxide and reading the optical density of the resulting solution at 550 nm with a microplate reader (MR 5000, Dynatech Laboratories, Embrach-Embraport, Switzerland). For each test condition, 4 specimens were used. Cytotoxicity was expressed as a percentage of the Teflon negative controls. Differences between groups were determined by analysis of variance with Tukey multiple comparison intervals ($\alpha = 0.05$).

Leakage study

Twenty extracted human canines and premolars without excessive root curvature (canal curvature 15-35°) were selected for this study. Each crown was sectioned below the cemento-enamel junction to obtain a 10 mm long root that was then prepared for endodontic treatment. During endodontic procedures, the canal space was mechanically enlarged using the Protaper endodontic files (Maillefer, Ballaigues, Switzerland) operated at 600 rpm under a constant irrigation with 3 % NaOCl (10 ml). The final preparation had a 6° taper and a diameter of 30 at the apex. The canals were then rinsed with distilled water (10 ml), dried with ethanol and paper points, and obturated with acrylic cones (Size M, Batch number: 0202 BF; Produits Dentaires, Vevey, Switzerland). Acrylic cones were used because pilot studies have shown that gutta-percha cones are auto-fluorescent when observed under the CLSM.

Leakage test

TRITC (tetramethylrhodamineisothyiocianate)-labeled lipopolysaccharide (LPS) was used as a marker for the leakage test. Briefly, stock solutions of TRITC were prepared by mixing 1.5 mg of TRICT-LPS (Sigma, Switzerland) with double-distilled water so that the final concentration of the solution was 0.01 mg/ml). Nail varnish was applied to the external part of the root to avoid the penetration of the tracer through dentin.

After 48 h, all specimens were rinsed with distilled water and embedded into cold curing epoxy (Epofix, Struers, CH). The specimens were then attached to the grips of a low speed saw (Isomet, Buehler Ltd, Lake

Bluff, IL, USA) and sectioned perpendicular to the tooth axis into 0.5 mm thick slabs. Each slab was mounted on microscopy glass slab and stored in dark until observation under the CLSM.

The sections were observed under a CLSM (LSM 510; Carl Zeiss, Jena, Germany) with two He- and Ne-lasers with excitation wavelengths of 543 nm and 633 nm, respectively. The presence of the TRICT-LPS inside the canal was detected with a HeNe green laser at 543 nm. Care was taken to keep the acquisition parameters (e.g., laser beam, pinhole, scan speed, pixel size) as constant as possible when comparing the different specimens.

Digital images of 564 x 564 pixels were recorded using a speed of 1.69 s/scan. For analysis, images were imported into Adobe Photoshop® (San Jose, CA), viewed at 200 % magnification, and the intensity of the rhodamine staining was viewed using the histogram function. For each image, the red-intensity (representing LPS-penetration) was viewed on the external root (maximum intensity), the center of the acrylic cone (minimum intensity), and at the dentin-sealer interface. The relative intensity of LPS-rhodamine leakage was estimated by dividing the intensity at the interface by the difference between the external intensity and that in the center of the cone. In this manner, each tooth served as its own control for intensity of red color. At the interface, the most intense color was selected for analysis on the basis that the worst area of LPS leakage is most clinically relevant.

The relative LPS penetration was averaged for five specimens for each type of material at three levels of sectioning: < 1 mm, 1-2 mm, and 2-3 mm from the apex. The mean LPS penetration for the three levels were compared by ANOVA and Tukey post-hoc comparisons ($\alpha = 0.05$). To compare

overall performance of the materials, the data for LPS penetration at all three levels were averaged (n = 15 for each material) and compared with ANOVA/Tukey analysis. The pooling of the data at different levels was an acceptable strategy because there were no statistical differences among the levels in the first analysis.

RESULTS

Cytotoxicity

For materials tested immediately after mixing (fresh condition), only the RSA exhibited low cytotoxicity (high SDH activity, Fig. 1). At 24 h, the RSA was statistically equivalent to the Tf controls (ANOVA, Tukey, α = 0.05). The PCS, TS, and REZ all suppressed SDH activity by > 60 % relative to the Tf negative controls, and all were statistically more cytotoxic that the RSA at all time points. All materials became more cytotoxic with time of exposure to cell-culture medium. The RSA, which showed little SDH suppression in the initial 24 h of testing, suppressed SDH activity of the Balb cells by as much as 35 % after 1 week. The PCS, TS, and REZ also became more cytotoxic with time, and all suppressed SDH activity by >90 % after 1 week.

When the materials were allowed to set for 24 h before testing (set condition), the results were similar to the fresh condition at 24 h, but all tended to be more stable, and there were few statistically significant increases in SDH suppression with time (Fig. 1). One exception was the REZ, which showed complete suppression of SDH activity at 48 h; this result may

have simply been aberrant. The RSA showed some tendency to increased SDH suppression with time, although differences were not statistically significant.

Sealing

The confocal technique was successful in measuring the sealing ability of the root canal sealers. On average, all materials showed significant leakage, but there was large variation among teeth (Fig. 2).

For each material, some teeth were nearly free of LPS-leakage (Figs 3a-6a), but LPS-rhodamine dye was detectable in nearly every tooth at some level, regardless of material (Figs. 3b-6b). Trends of leakage of LPS as a function of distance from the apex were not consistently demonstrable for any material or tooth (Fig. 2). Overall, the RSA material was more effective in sealing root canals against LPS leakage than any other material (Fig. 2, statistically significant, ANOVA, Tukey, $\alpha = 0.05$).

DISCUSSION

The biocompatibility of endodontic sealers is of particular importance because these materials are in direct contact with the periapical tissues and any degradation products might elicit adverse effects (BARTHEL et al. 1999). The results of the current study show that all classes of currently available endodontic sealers pose significant biological risks, particularly in the freshly mixed condition (Fig. 1). When first mixed all the materials except the RSA were severely cytotoxic, and all, including RSA, increased in cytotoxicity with time. The increasing toxicity with time is in contrast to other ma-

terials, including composites (WATAHA et al. 1999), alloys (NELSON et al. 1999), and ceramics (MESSER et al. 2003). The tendency to increasing cytotoxicity with time was not observed for materials that had set 24 h (Fig. 1). This could indicate that set materials released less toxic components in the cell culture medium. However, the freshly mixed condition is relevant to clinical use because the sealers are placed into the canal unset and must set in situ. Thus, it appears that the biological risks of sealers are relatively high compared to other dental materials currently available, and that there is much room for improvement in this regard.

Of the materials tested in the current study, RSA was the least cytotoxic (Fig. 1), and was markedly better than the other sealers—which represented the major classes of sealers available in clinical endodontic practice today. This result is in agreement with previous reports (OZTAN et al. 2003). Although the cytotoxicity of the RSA was not ideal, it represents an improvement over these other materials, and is more compatible with contemporary restorative materials that cannot tolerate eugenol or must be compatible with polymerization chemistries (TJAN & NEMETZ 1992).

The confocal microscopic technique used in the current study to assess LPS leakage was successful and represents an improvement over alternatives such as light microscopic viewing of basic fuchsine, methylene blue, silver nitrate, or other biologically irrelevant dyes (HILTON 2002). A key advantage of the confocal technique is its ability to ‘see’ below the surface and avoid polishing artifacts that overestimate the penetration of dyes. Fur-

thermore, the covalent bond between the LPS and rhodamine dye was nearly ideal since the rhodamine cannot diffuse without LPS, and the rhodamine is much smaller than the LPS, so that its presence probably does not reduce dye penetration. The controls for the confocal technique also were promising, because the design provided an internal standard for dye penetration (the external root fluorescence and the canal core). Other advantages included a high sensitivity of the fluorescence and control over many important variables, such as time of exposure, temperature, and canal preparation technique. The use of nail varnish to prevent penetration of LPS by lateral canals appeared successful because several sections (Figs 3A, 4A, 5A, 6A) did not have evidence of staining. This observation is in agreement with previous reports (LAGHIOS et al. 2000).

The experimental design of the leakage test represented a 'worst case' scenario for canal sealers. Acrylic points were used to condense the sealers inside the canals because gutta-percha points fluoresce. Because gutta-percha is more deformable, successful condensation of the canals would have been better than achievable in the current study. However the acrylic cones used in the current study were calibrated cones with the same conicity than the mechanical files (all had a 6° taper). The current study used the highest degree of dye penetration (highest LPS-rhodamine flluorescence) to estimate LPS penetration. Although this type of design may overestimate toxicity or leakage, it is biologically relevant because it is the maximum concentration of LPS to which the tissues will react in vivo.

No sealer in the current study successfully sealed the apex against LPS penetration. Furthermore, all materials were highly variable in

their ability to seal the root canal (Figs 3 to 6). These issues reaffirm the difficulties in preparing and condensing a filling in endodontics. Factors such as canal preparation, cleaning, and disinfecting or anatomy of the canal system were probably all that influenced the ability of the sealers to prevent LPS penetration (CHAVEZ DE PAZ et al. 2003). The RSA material was the most promising in this regard (Fig. 2) because leakage was less and less variable among teeth.

The biological risk of using an endodontic sealer is critically dependent on both the cytotoxicity of the material and its ability to seal the root canal. Ideally a material would have minimal cytotoxicity and would allow no leakage. Then the ability of degradation products to be created and diffuse into the periapical tissues would be least under these conditions. Of the materials tested in the current study, the RSA (a silicone-based material) was by far the most promising because it had low cytotoxicity and good ability to seal the root canal (Figs 1-2). However, even the RSA material was not ideal and the current results support the need to continue to develop better materials from a biological perspective. The ability of eugenol-based sealers like PCS to perform well in vivo may be due to inadvertently favorable effects of the eugenol. PCS was severely and persistently cytotoxic and exhibited significant LPS leakage (Fig. 6B). However, the eugenol may reach sufficient concentrations to kill bacteria which leak into or through the canal (HUME 1988). In spite of the ability of these types of materials to perform well in vivo, better materials with lower cytotoxicity and leakage would be more desirable. Resin sealers are particularly troubling in endodontics because they are hard to remove when set. If bonded to the canal, they experience tremendous in-

ternal polymerization stresses because of unfavorable C-factors that lead to debonding and leakage (BOUILLAGUET et al. 2003). Further the irregular and variable structure of the apical region of human teeth represent special challenges during endodontic therapy and therefore obturation techniques based on the penetration of resins into dentinal tubules are unlikely to be successful (MJÖR et al. 2001). More work is definitely needed to confirm these preliminary *in vitro* results

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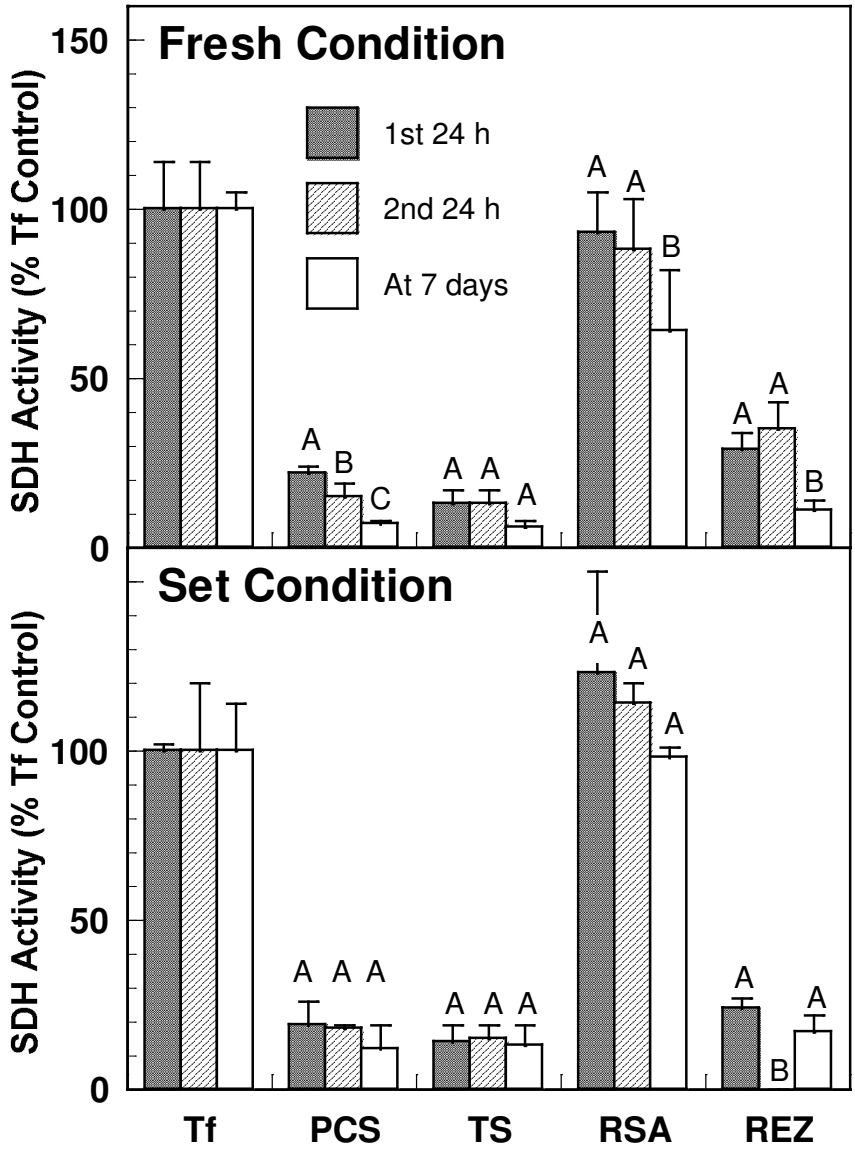
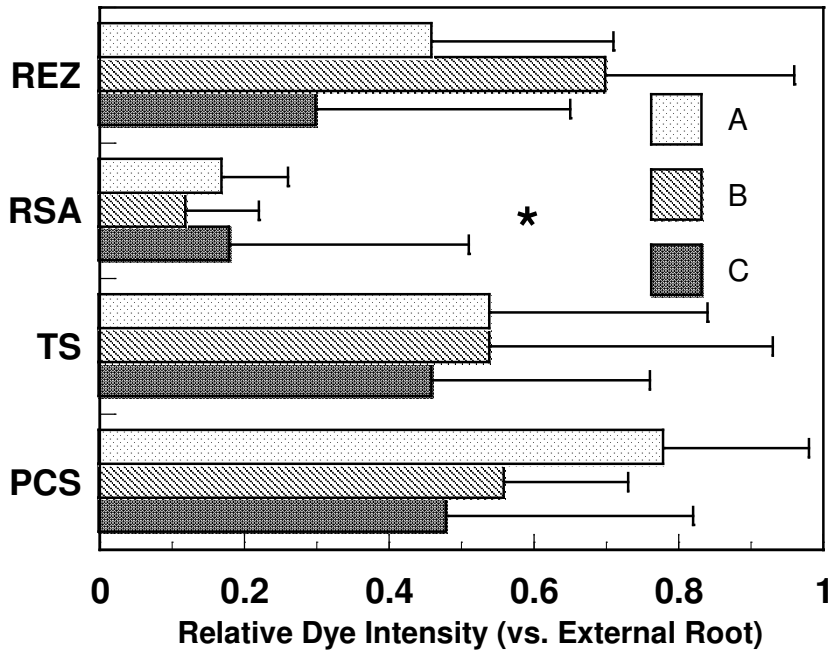
Figure 1



Cellular response to endodontic sealers placed immediately in contact with cultured cells (Fresh condition) or 24h after setting (Set Condition). initially (1st 24h), after 24h (2nd 24h) and after one week of strorage in sterile PBS (At 7 days). Cellular response was measured in terms of succinic dehydrogenase (SDH) activity, expressed in terms of Teflon (Tf) negative controls. Error bars indicate 1 standard deviation of the mean (n=4). Within each set of columns, different letters indicate statistical differences (ANOVA, Tukey, α =0.05).

Figure 2



LPS-rhodamine penetration inside the root canal at level A (< 1 mm from the apex), level B, (1-2 mm from the apex) and level C (2-3 mm from the apex). The relative intensity of LPS-rhodamine leakage was estimated by dividing the intensity of the rhodamine at the interface between the sealer and the acrylic cone by the difference between the external intensity and that in the center of the cone. Because there was large variations among teeth, ANOVA and Tukey post-hoc comparisons (α = 0.05) did not show statistical differences between the three locations from the apex. The RSA material was more effective in sealing root canals against LPS leakage than any other material (statistically significant, ANOVA, Tukey, α = 0.05).

Figures 3 A and 3 B

Endo Rez specimens. Confocal microgaph (3A) showing a section of the root canal treatement after the leakage test (original magnification X 10). The micrograph shows the presence of rhodamine-LPS at the external surface of the root (top left) and the absence of dye penetration (arrows) at the interface between the sealer and the acrylic cone whereas the rhodamine-LPS dye has penetrated into the porosities of the resin sealer (3B).

Figures 4 A and 4 B

RSA Roeko Seal specimens. The CLSM images (original magnification X 10) show no penetration of rhodamine-labeled LPS (arrows) in micrograph 4A and the presence of dye around the acrylic cone in micrograph 4B.

Figures 5 A and 5 B

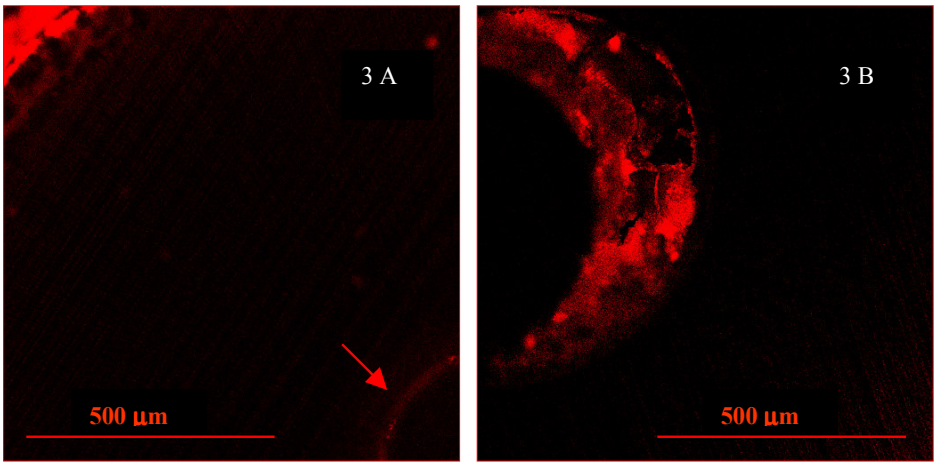
Top Seal specimens (original magnification X 10). Confocal micrograph 5A shows the presence of rhodamine-LPS at the external surface of the root (top left) and the absence of dye penetration (arrows) at the interface between the sealer and the acrylic cone. In micrograph 5B, the dye is observable in the isthmus of the root canal exhibiting a defective sealing.

Figures 6 A and 6 B

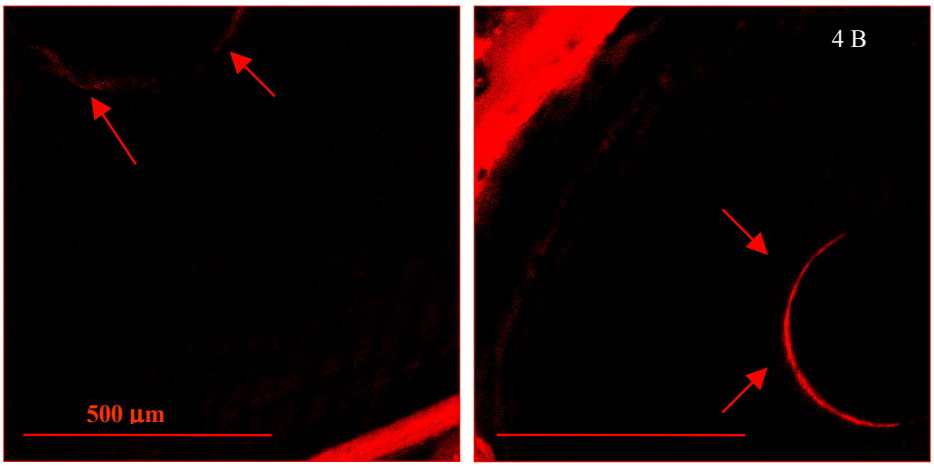
PCS specimens (original magnification X 10). Micrograph 6A shows the presence of rhodamine-LPS at the external surface of the root (bottom left)

and the no rhodamine-LPS penetration (arrows). In micrograph 6B, the dye is observable at the interface between the sealer and the dentin.

REZ



RSA



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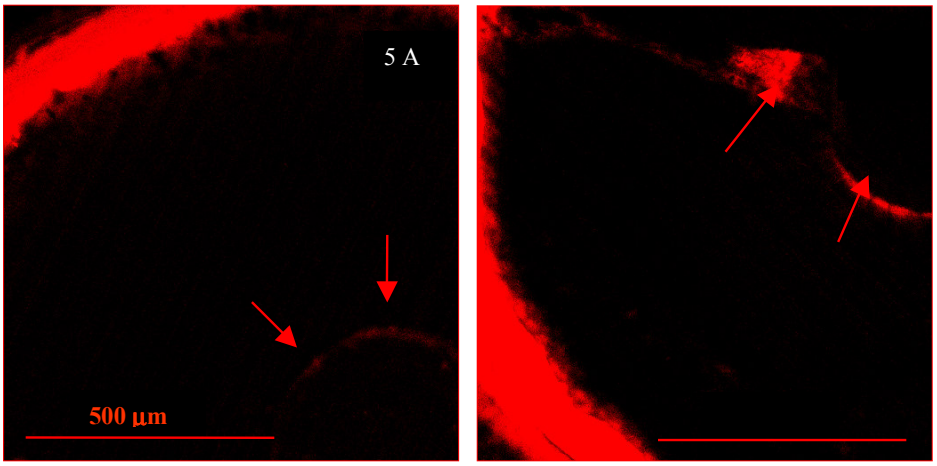

PCS