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Research Article

Reaction of Rat Subcutaneous Connective Tissue to an Experimental Endodontic Sealer

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Abstract

Aim of the Study: The purpose of this study was to evaluate the reaction of subcutaneous connective tissues in rats to an experimental endodontic root canal sealer with MCP (ERCS) and AH-Plus (AHP), a well-established root canal sealer.

Methods: Sterile medical-grade silicone tubes containing the test materials were implanted subcutaneously in 30 Wistar rats. After 10, 30 and 90 days the animals (n = 10 per period) were euthanized and the implants along with their surrounding tissues dissected, fixed and processed for histologic evaluation. A four-category evaluation system was used to measure and record the microscopic observations; the occurrence and thickness of a fibrous capsule, the vascular changes and the various types of inflammatory cells. The tissue response adjacent to the lateral walls of the silicone tubes (LWSt) served as negative control.

Results: Initially a severe inflammatory reaction was observed in direct contact with both test materials. The severity of the ERCS reaction had decreased at the 30-day period and no inflammatory reaction was observed at the end of the experiment. AHP after 30 days showed persisting inflammatory cells in contact with the material. The inflammatory reaction had decreased after 90 days, however, isolated inflammatory cells were still present in the surrounding tissues. The LWSt did not show adverse reactions at any time period during the experiment.

Conclusion: At the end of 90 days the ERCS demonstrated biocompatibility when implanted in subcutaneous connective tissue in rats, while AHP remained slightly toxic even after 90 days.

Keywords: *Biocompatibility; Endodontics; Resin-Based Sealers; Tissue Response*

Introduction

The current concept among clinicians is that after debridement and disinfection, complete obturation of the root canal space with a biocompatible/bioactive material constitutes the key to successful endodontic therapy [1]. Historically, different materials have been advocated for filling root canals while gutta-percha and a sealer cement are the most widely used [2,3]. During the last 20 years, resin-based and calcium silicate-based materials have gained popularity and are universally being used for root canal obturation [4-10]. Previous reports have shown that these biocompatible/bioactive endodontic sealers are well tolerated by the living tissues and have shown promise for *in vivo* human clinical trials [11-13].

Recently, an experimental endodontic sealer with MCP (ERCS, Pulpdent, Watertown, USA) has been developed. According to the manufacturer, ERCS is a radiopaque bioactive dual-cure material comprised of a mixture of aliphatic urethane dimethacrylate and other aliphatic dimethacrylates. The resin matrix also contains a hydrophilic phosphoric acid monomer which imparts hydrophilicity and promotes good marginal adaptability and penetration into the dentinal tubules. A rubberized urethane methacrylate present in the resin promotes adhesion to gutta-percha, while in the presence of moisture the modified methacrylate calcium phosphate (MCP) imparts bioactivity, resulting in apatite deposits at the sealer/dentine interface [14], thus improving the sealing properties of the material [15]. Tested according to ISO 4049 (Skaria., *et al*. 2021;

Unpublished data), the material exhibited a high radiopacity (equivalent of 3.2 mm Aluminum), which is achieved through the addition of 67% inert barium glass and barium sulphate. Solubility data showed a mean water absorption of 0.94% (0.01). The ERCS comes in a double-barrel syringe and the material can be injected by means of an auto mixing tip directly into the root canal. The sealer has a working time of more than 10 minutes and will set completely in 2h under anaerobic conditions. After polymerization, the material demonstrated good dimensional stability when stored in water over a period of 6 months (mean dimensional stability = 0.78% [0.15]). The light curing feature of the dual cure material offers coronal polymerization to a depth of 3mm, which allows completion of the coronal restoration at the same appointment.

To date, there have been no studies reported evaluating the biocompatibility of ERCS.

Purpose of the Study

The purpose of this study was to evaluate the biocompatibility of ERCS and compare it to a with a well-established resin-based endodontic sealer i.e. AH-Plus (AHP; Dentsply, De Trey GmbH, Konstanz, Germany) when implanted into the subcutaneous connective tissues of rats. The null hypothesis tested was that there are no significant biocompatibility difference between ERCS and AHP.

Materials and Methods

Sample preparation and implantation

The protocol of this study received approval from the Institutional Research Ethics Committee of the Argentine Dental Association (Code #0121/2021-2, AOA). Sixty autoclaved medical grade silicone tubes closed at one end (Raholin SRL, Villa Madero, Buenos Aires, Argentina) and measuring 10 mm long with an internal diameter of 1 mm and external diameter of 2 mm were divided into two groups of 30 (n = 30) tubes each and filled flush at the open end with freshly prepared ERCS or AHP. The lateral walls of the silicone tubes (LWSt) served as negative controls. Care was taken not to overfill or smear the LWSt with the test materials. The sealers/groups were prepared under sterile conditions and according to the manufacturers´ recommendations. Sample preparation was as follows:

- **Group 1 ERCS (n = 30):** The material was expressed from the auto mixing tip of the two-barrel syringe onto 4.0 X 4.0 mm sterile silicone dishes (Raholin SRL). The freshly mixed ERCS was then transferred to the silicone tubes using size #35 K-Files (Maillefer/Dentsply, Ballaigues, Switzerland) and immediately placed subcutaneously.

- **Group 2 AHP (n = 30):** The sealer was prepared onto a sterile glass slab under normal room conditions and then transferred to the silicone tubes using the same procedures as in group 1.

In both groups, excess material was removed with a sterile spatula. After preparation, the samples were immediately implanted into the subcutaneous connective tissue of 30 white male Wistar rats weighing approximately 250g each. The husbandry and management of the animals met the requirements of ISO 10993-1 and 10993-2, (1992) standards [16,17], as well as the International Regulatory Requirements for the care and use of laboratory animals [18]. Every effort was made to minimize animal discomfort and limit the total number of animals used. All operative procedures were done under strict aseptic conditions.

Implantation of the test materials was as follows. After anesthesia through intraperitoneal administration of ketamine chloride and acepromazine (14 mg/10 mg/Kg body weight) the dorsal skin was shaved and disinfected with 5% iodine in alcohol. An incision of approximately 18 mm was made through the skin and two separate dorsal pockets were prepared by blunt dissection. They were amply separated from one another to avoid cross contamination. ERCS and AHP (one sample of each per animal) were gently placed into the pockets of each rat to a depth of 20 mm from the line of the incision. Finally, the wounds were closed with silk sutures. The animals were maintained in cages and fed a regular diet with water *ad libitum*. They were euthanized in groups of 10 each after 10, 30, and 90 days with an anesthetic overdose.

Histologic preparation and evaluation

The implants along with their surrounding tissues were carefully dissected and immediately fixed in 10% neutral buffered formalin (pH 7.4). After fixation the tissues were processed for paraffin embedding. The paraffin blocks were oriented parallel to the long axis of the tubes and longitudinal semi-circular sections of approximately 7-µm thick were obtained from the central areas

of the implants. The sections were stained with hematoxylin and eosin. To estimate the tissue response in the areas of tissue/material contact, three sections belonging to the central areas of each specimen were analyzed and photographed at different magnifications with a photomicroscope (Carl Zeiss, Oberkochen, Germany). The sections were blindly analyzed by two trained evaluators who independently scored the tissue reaction using the following four criteria: 1. No reaction: fibrous-capsule formation and absence of inflammatory cells; 2. Mild reaction - presence of a fibrous tissue formation with few remaining inflammatory cells; 3. Moderate reaction - fibrous tissue formation with the presence of concentration of polymorphonuclear leukocytes, lymphocytes, plasmocytes and macrophages; 4. Severe reaction - presence of large accumulations of polymorphonuclear leukocytes, lymphocytes, plasmocytes, macrophages, foreign-body giant cells and congested capillaries. The evaluators had been calibrated previously by analyzing a set of 80 similar, but unrelated slides showing different types of inflammatory reactions to endodontic sealers. If there was a disagreement between the evaluators, the sample under discussion was analyzed jointly until a consensus was reached. In order to standardize the number of the total analyzed samples, only 10 (n = 10) randomly selected LWSt samples (five from each group) per period were evaluated for tissue reaction. Data was analyzed by the Wilcoxon Signed Rank test to determine if there was a statistically significant difference between ERCS and AHP at each observation period. The total effect of time and material upon the tissue reaction was calculated by the Kruskal-Wallis and the Dunn's test. The significance level was set at $p < 0.05$. At the end of the experiment the sealers were considered biologically acceptable if the tissue reaction was recorded as 1 or 2. Due to a procedural error one animal from the 10-day subgroup had to be excluded from the study and replaced by another one in which the same implantation procedures were carried out.

Results

Macroscopic examination at the implantation sites revealed that wound healing was uneventful at all observation periods. The number of implants and the severity of tissue reaction to materials is presented in table 1.

Days	n	ERCS				AHP					LWSt		
		1	2	3	4	1	2	3	4	1	2	3	4
10	10	-	-	-	10	-	-	-	10	7	3	-	-
30	10	-	10	-	-	-	1	9	-	10	-	-	-
90	10	7	3	-	-	-	2	8	-	10	-	-	-

Table 1: *Tissue reaction to materials.*

1: No Inflammatory Reaction; 2: Mild Reaction; 3: Moderate Reaction; 4: Severe Reaction.

Tissue reaction to ERCS and AHP

After 10 days the tissue reaction of ERCS and AHP was severe and prominently present at the open end of the silicone tubes (Figure 1A-1D). When in direct contact with the material the majority of ERCS and AHP samples exhibited an inflammatory reaction that was located at the slight invagination of the material in the lumen of the tubes. Some AHP samples had necrotic tissue in direct contact with the material. In both groups there were many randomly dispersed dark particles which appeared to have been released from the test materials.

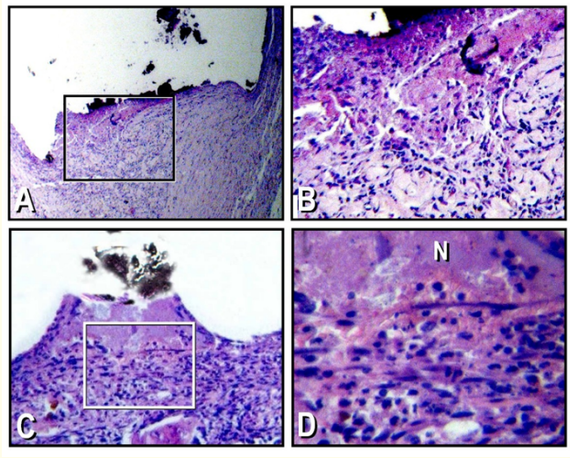

Figure 1A-1D: *Representative specimens of ERCS and AHP at the 10-day observation. A: ERCS. Overview of the area tissue/material contact (H&E, Original magnification X40). B: Higher magnification of outlined area in A. There is a severe granulomatous tissue reaction containing dark particles of the test material (H&E, Original magnification X100). C: AHP. At the area of tissue/material contact note a slight invagination of the tissue into the lumen of the tube with a necrotic area with adjacent severe granulomatous tissue (H&E, Original magnification X40). D: Higher magnification of outlined area in C. There is a thick necrotic tissue in direct contact with the sealer (N). Below it, a severe granulomatous tissue reaction containing many lymphocytes, polymorphonuclear leucocytes and fibroblasts (H&E, Original magnification X400).*

At the 30-day observation period, the intensity of the inflammatory reaction in contact with ERCS was scored as mild. The tissue reaction to AHP was scored as moderate in nine instances while one case revealed a mild reaction (Figure 2A-2D). In direct contact with both materials there was a fibrous tissue reaction containing some dispersed material particles and many newly formed blood vessels. For the AHP samples a persistent granulomatous tissue was observed.

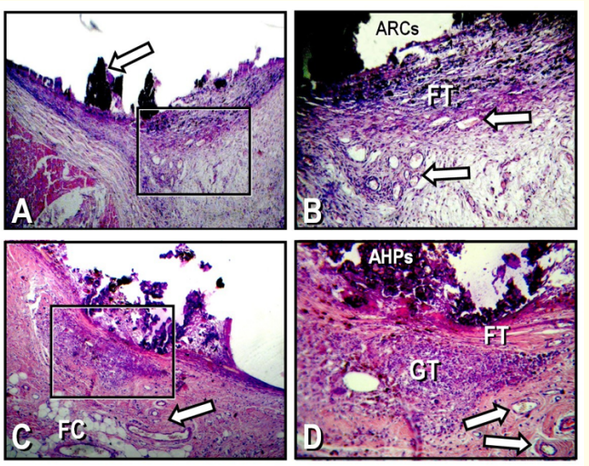

Figure 2A-2D: *Representative specimens of ERCS and AHP at the 30-day observation. A: ERCS. Overview of the area of tissue/material contact (arrow). There is a fibrous connective tissue containing many blood vessels (H&E, Original magnification X40). B: Higher magnification of outlined area in A. In direct contact with the sealer (ERCS), dense fibrous tissue (FT) can be seen. Below it, many newly formed vessels (arrows) surrounded by inflammatory cells are present (H&E, Original magnification X150). C: AHP: Overview of the area of tissue/material contact. There is a fibrous connective tissue containing many particles of material, blood vessel (arrow) and a persisting area of chronic inflammatory cells. FC: Fat cells. (H&E, Original magnification X40). D: Higher magnification of outlined area in C. In direct contact with the sealer (AHP) there is a thick band of fibrous connective tissue (FT) containing many material particles. Below it, a persisting granulomatous tissue (GT) and wide newly formed capillaries (arrows) can be seen (H&E, Original magnification X150).*

At the 90-day observation period the majority of ERCS samples scored no reaction. In only three samples the tissue reaction was categorized as mild. When in direct contact with the material, a reparative dense fibrous tissue had formed, which contained a few remaining lymphocytes (Figure 3A and 3B). However, when AHP was in direct contact with tissues, the fibrous tissue that had formed still contained persisting inflammatory cells. The reaction scores to the material was mild (2 samples) and moderate (8 samples). (Figure 3C and 3D).

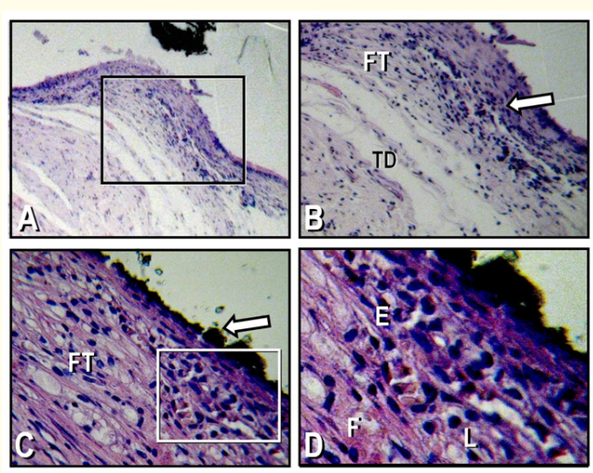

Figure 3A-3D: *Representative specimens of ERCS and AHP after 90 days. A: ERCS: Overview of the area of tissue/material contact showing remnants of the sealer and a fibrous tissue in the process of reparation. Note the presence of inflammatory cells that still need to be resolved (H&E; Original magnification X40). B: Higher magnification of outlined area in A. In contact with the material note the reparative process showing dense healthy fibrous connective tissue (FT) and a few remaining inflammatory cells (arrow), mostly lymphocytes (H&E; Original magnification X100). C: AHP: Overview of the area of tissue/material contact (Arrow). There is fibrous connective tissue (FT) that still contains remaining inflammatory cells (square area) (H&E; Original magnification X100). D: Higher magnification of outlined area in C. Note the presence of lymphocytes (L), a few polymorphonuclear leucocytes, fibroblasts (F) and eosinophils (E) (H&E; Original magnification X850).*

Negative controls

After 10 days, the tissue reaction to the LWSt showed a healthy thin connective tissue in the majority of the samples. Only three of the LWSt showed few dispersed inflammatory cells. At the 30-day observation period the thickness of the fibrous connective tissue had increased progressively and without inflammatory cells.

At the end of the experiment the LWSt reacted in a similar fashion as in the 30-day period. By applying the Wilcoxon Signed Rank test, no statistically significant differences ($p > 0.05$) were found between ERCS and AHP at the 10-day observation period. After 30 days, significant differences were found between ERCS and AHP ($p < 0.05$). In both periods (10 and 30 days) ERCS and AHP significantly differed from LWSt ($p < 0,05$). At the 90-day observation period, ERCS still showed significant differences with AHP ($p < 0,05$) but not with LWSt ($p > 0.05$), while AHP was significantly different from LWSt ($p < 0,01$). The total effect of time for ERCS and AHP revealed that the results obtained at 10 and 30 days significantly differed ($p < 0.05$) with those obtained at the 90-day observation period. Conversely, no significant differences ($p > 0.05$) were observed for LWSt between all time intervals. Therefore, the null hypothesis was rejected.

Discussion

Implantation of endodontic filling materials into subcutaneous connective tissue of the rat is considered a valid secondary screening test for biocompatibility [16,17]. The implantation periods used in this study were within the normal short and long-term intervals of the recommended Standard Practices for Biological Evaluation of dental materials [18]. Resin-based AHP sealer was used for comparison because its toxicity has been previously determined by *in vitro* [19,20] and *in vivo* experiments [21-23]. Medical grade silicone tubes were used as carriers for the test materials because the tubes by themselves have proven biocompatibility [24,25]. The implantation method brings the sealers into immediate contact after mixing with connective tissues, which simulates what may occur at the apex; contact with periapical tissues after root canal obturation.

With regards to the test materials it should be noted that ERCS is a novel experimental formulation, which has not previously been tested for biocompatibility. Thus, the evaluation of its biological reaction is essential to provide relevant data on its safety for clinical use. In order to minimize the formation of an oxygen inhibited layer of the ERCS samples, they were upon filling immediately implanted in the tissues. Oxygen inhibits free-radical polymerization of resin components yielding an incomplete setting of the sealer. This is of concern because unpolymerized material will alter its biological response. The further elution of uncured components from the oxygen inhibited layer may then generate a false biological response that does not represent the true properties of the material.

Severe earlier reactions after implantation were observed for ERCS and AHP demonstrating that even after 10 days they still caused irritation to the tissues. The initial aggressive reaction caused by AHP was not unexpected and is in agreement with reports by Grecca., *et al.* [21] and Simsek., *et al* [22]. The surgical trauma during the placement of the implants could be another factor that contributed to the early tissue reactions. However, the severity of the inflammatory reaction for both sealers decreased over time and was resolved at the end of the experiment (90-days) for ERCS, while a few persistent inflammatory cells were still present in tissues in contact with AHP. This finding that was also reported in previous investigations [23,26,27].

Conclusion

Within the limitations of this study, it was concluded that ERCS demonstrated biocompatibility and was well tolerated by the subcutaneous connective tissue of the rat after 90 days. After 90 days AHP remained slightly toxic. Clinical studies in humans are indicated to confirm these findings and demonstrate clinical efficacy.

Conflict of Interest

The authors declare that they have no conflict of interest.

Disclosure

This paper or any part of it, has not been submitted or published elsewhere while being considered by the *Scientific Archives of Dental Sciences*.

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