Cytotoxicity of Mineral Trioxide Aggregate Using Human Periodontal Ligament Fibroblasts

Karl Keiser, DDS, MS, C. Chad Johnson, BS, and David A. Tipton, DDS, PhD

The purpose of the present study was to compare the cytotoxicity of mineral trioxide aggregate (MTA) to other commonly used retrofilling materials, Super-EBA and amalgam. This was accomplished using a cell viability assay for mitochondrial dehydrogenase activity in human periodontal ligament fibroblasts after 24-hr exposure to extracts of varying concentrations of the test materials, in both freshly mixed and 24-hr set states. Methyl methacrylate 2% (vol/vol) served as the positive control, and complete culture medium served as the negative control. Differences in mean cell viability values were assessed by ANOVA (p < 0.05). In the freshly mixed state, the sequence of toxicity was amalgam > Super-EBA > MTA. In the 24-hr set state the sequence of toxicity at a low extract concentration was Super-EBA > MTA, amalgam, and Super-EBA > amalgam > MTA at a higher extract concentration. This study supports the use of MTA in the root-end environment.

Several retrospective studies have addressed the outcome of conventional, nonsurgical root canal therapy, and the success rates reported have ranged from 95% (1) to 53% (2). Therefore, a certain number of failures can be expected, likely due to the persistence of bacteria and their byproducts in the root canal system. Although many endodontic failures can be successfully retreated by an orthograde approach (3), root-end surgery may be the only alternative to extraction in cases where the orthograde approach is not feasible due to the presence of posts and other permanent restorative materials in the coronal aspect of the root canals.

The repair processes that take place in the periradicular tissues after root-end surgery have been well documented (4–6). Healing of the osseous excisional wound involves regeneration of trabecular bone, with the reformation of a functional periosteum and cortical plate. The ultimate success of the surgery depends on the regeneration of a functional periodontal attachment apparatus, including cementum overlying the resected root-end surface, periodontal ligament (PDL), and alveolar bone (7). This would occur more predictably when the root canal that is exposed after root-end resection is filled with a material that not only seals the canal to

prevent egress of any remaining bacteria or their byproducts, but also allows for the formation of a normal periodontium across its exterior surface.

Materials that have been advocated for use as retrofilling agents include amalgam, composite resin, zinc oxide-eugenol cements, ethoxybenzoic acid (EBA) cements, glass ionomer cements, polycarboxylate cements, Cavit, and gutta-percha, among others (8). To date, none of these materials has been shown to allow predictably for the regeneration of a normal periodontium across the entire resected root surface. Thus the need still exists for a biocompatible material that adequately seals the exposed root canal system after root-end resection and that permits the periapical tissues to heal in an ideal manner via regeneration of cementum, PDL, and alveolar bone.

Recently, an experimental material, mineral trioxide aggregate (MTA), has been proposed for use as a retrofilling agent (9). Its ability to seal a resected canal system was assessed in vitro (10), and when used as a root-end filling material in dogs, cementum was reported to have formed over its exposed surface (11). When compared with standard root-end filling materials, the cytotoxicity of MTA was found to be less toxic than IRM or Super-EBA, but more toxic than amalgam (12). This investigation used an immortalized mouse fibroblast line and assays that determined the integrity of the cell membranes and the uptake of neutral red dye to assess cytotoxicity.

The purpose of the present study was to further investigate the cytotoxicity of MTA as compared with Super-EBA and amalgam, using human PDL fibroblasts, and an assay that assessed the metabolic activity of cells after exposure to extracts of the test materials.

MATERIALS AND METHODS

Cell Culture

PDL fibroblasts were obtained from the roots of impacted human maxillary third molar teeth extracted in the Oral Surgery Clinic at the University of Tennessee College of Dentistry. The following procedure was adapted from Somerman et al. (13).

Immediately after extraction, the teeth were placed in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Inc., Grand Island, NY) at 4°C. PDL tissues attached to the middle third of the roots were gently curetted off and placed in DMEM containing streptomycin (100 μg/ml), gentamycin (50 μg/ml), and

amphotericin-B (250 μg/ml) (Sigma, St. Louis, MO) to prevent contamination. These tissues were minced and rinsed twice in DMEM, then allowed to attach to the bottom of 12.5 cm^2 tissue culture flasks. DMEM supplemented with 20% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and antibiotics (complete DMEM) were added to the flasks followed by incubation at 37°C in a humidified atmosphere of 5% carbon dioxide–95% air until fibroblast-like cells had grown to confluency. Confluent primary cultures were trypsinized (0.25% trypsin) (Life Technologies, Inc.), collected by centrifugation (10 min at 1200 rpm), resuspended in complete DMEM, and subcultured in 75 cm^2 flasks (Becton Dickinson, Franklin Lakes, NJ). After reaching confluency, first passage cells were trypsinized, collected by centrifugation, placed in a freeze medium (DMEM complete, supplemented with 10% dimethyl sulfoxide (DMSO); Pierce, Rockford, IL), and stored in liquid nitrogen until needed; cells from the second to fourth passages were used in the cytotoxicity experiments described herein.

Test Material and Extract Preparation

Test materials used were MTA (Loma Linda University, Loma Linda, CA), Super-EBA (Harry J. Bosworth, Skokie, IL), and a dispersed phase amalgam (Dispersalloy Dentsply Caulk, Milford, DE). Methyl methalcrylate (MMA) 2% (v/v) was used as a positive control (14). The materials were mixed according to the manufacturers' recommendations and placed into the bottom of 48-well tissue culture plates (Becton Dickinson) to achieve a thickness of ~5 mm. The surface area of the test material exposed was 64 mm^2. These samples were divided into two groups. The first group included all materials in a freshly mixed state, whereas in the second group, the materials were allowed to set for 24 hr at 37°C at 100% relative humidity.

Extracts of the test materials were made as follows: 200 μl of complete DMEM was placed over each sample (ratio of surface area exposed to volume of extract vehicle = 64 mm^2/200 μl = 320 mm^2/ml), and the plates were incubated at 37°C at 100% relative humidity for 24 hr. The medium was then drawn off and sterile-filtered at 0.22 μm. To observe a dose–response relationship, the extracts were serially diluted 1:1 with complete DMEM to achieve a total of five concentrations of each extract that were then tested for cytotoxicity as described. MMA 2% (v/v) was dissolved in complete DMEM and tested as positive control. Complete DMEM placed into empty 48-well tissue culture plates for 24 hr was tested as negative control.

Cytotoxicity Testing

The following protocol is adapted from Schweikl and Schmalz (14) and Wataha et al. (15). Single cell suspensions of human PDL fibroblasts were seeded in 96-well flat-bottomed plates, 5 $\times$ 10^3 cells per well as determined by hemocytometer counting, in complete DMEM, and incubated in a humidified atmosphere of air and 5% CO$_2$ at 37°C for 24 hr. The culture medium was then replaced with 200 μl aliquots of the test extracts or control media, and the cells thus exposed were incubated for 24 hr at 37°C under humidified air and 5% CO$_2$. Five wells were used for each single extract concentration. After exposure, cell viability was determined by the ability of the cells to cleave the tetrazolium salt (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromid) (MTT) to a

formazan dye, using a kit from Boehringer-Mannheim Corp. (Indianapolis, IN); only metabolically active cells are able to do this. The medium was removed with a sterile pipette, and 200 μl of phosphate-buffered saline was added to each well, swirled gently for 1 min, then replaced with 100 μl of complete medium and 10 μl of a 5 mg/ml solution of MTT. The cells were incubated for 4 hr at 37°C in a 5% CO$_2$ atmosphere in the MTT/medium solution. One hundred microliters of a 6.25% vol/vol 0.1 mol/L NaOH in DMSO solution was added to each well, and the plates were incubated overnight to solubilize any formazan crystals that had formed. Plates were shaken for 60 min at room temperature on a plate shaker to achieve uniform color. Optical densities were then measured at 570 nm in a multiwell spectrophotometer.

Data Analysis

Mean absorbance values obtained from the DMSO-solubilized formazan for each extract concentration were calculated and expressed as a percentage of the mean negative control value (set at 100% viability). Differences in mean cell viability values between materials were assessed by one-way ANOVA and Scheffé's post-hoc test ($p \leq 0.05$).

RESULTS

The highest extract concentration for all materials gave cell viability values that were not significantly different from the positive control. The two lowest concentrations tested gave cell viability values that were not significantly different from the negative control. Therefore, the first (labeled "high") and second (labeled "low") serial dilutions proved to be the most appropriate for the detection of toxicity using the MTT assay and human PDL fibroblasts. Figures 1 and 2 show cell viability relative to extract dilution for fresh and 24-hr set samples, respectively.

In the fresh sample group, at the lower concentration, the sequence of toxicity was amalgam > Super-EBA > MTA ($p <$ 0.0001). At the higher concentration, the sequence of toxicity was amalgam, Super-EBA > MTA ($p < 0.0001$). There was no significant difference between amalgam and Super-EBA.

In the 24-hr set sample group, at the lower concentration, the sequence of toxicity was Super-EBA > MTA, amalgam ($p <$ 0.0001). There was no significant difference between MTA and amalgam. At the higher concentration, the sequence of toxicity was Super-EBA > amalgam > MTA ($p < 0.0001$).

Amalgam proved to be less toxic after 24 hr at both concentrations. The toxicity of Super-EBA was not significantly different after 24 hr. The toxicity of MTA dropped significantly after 24 hr at the higher concentration only.

DISCUSSION

A variety of test systems are available to determine the cytotoxicity of dental materials in cultured mammalian cell populations (14). Permeability assays monitor the integrity of cell membranes by the inclusion or exclusion of vital dyes, or by the release of radiolabeled chromium. Replication assays indirectly assess the ability of cells to proliferate by measuring the incorporation of nucleotide analogues that have been radiolabeled or are detectable by immunoassay during DNA synthesis. Changes in the cellular cytoskeleton or at the cell surface are observed by morphological
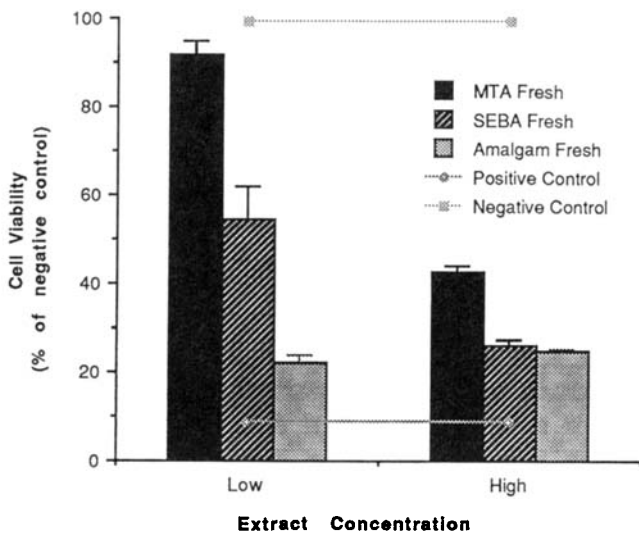
FIG 1. Mean cell viability (plus one standard deviation) at low and high extract concentration using freshly mixed materials. MMA 2% (v/v) used as positive control. Complete culture medium used as negative control. SEBA = Super-EBA.
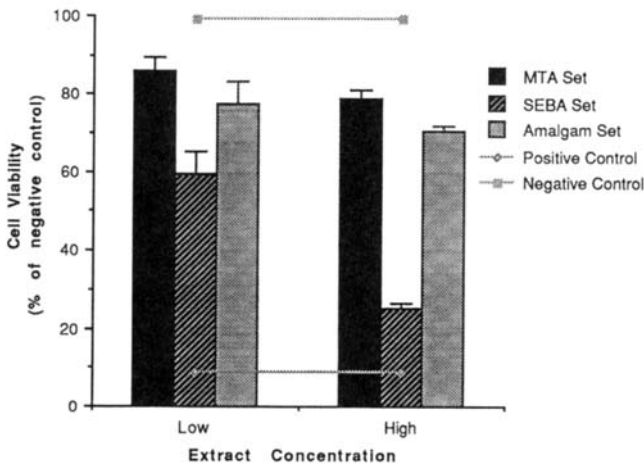

FIG 2. Mean cell viability (plus one standard deviation) at low and high extract concentration using 24-hr set materials. MMA 2% (v/v) used as positive control. Complete culture medium used as negative control. SEBA = Super-EBA.

studies. Finally, functional assays typically evaluate the cell's ability to provide the energy necessary for anabolic activities, or the endproducts of such activities. The assay used in the present study used the tetrazolium salt MTT to measure mitochondrial dehydrogenase activity (16). It is a pale yellow substrate that produces a dark blue formazan product when cleaved by active mitochondria, and so the reaction only occurs in living, metabolically active cells.

The decision to use a particular test system should be based on its consonance with the chemical nature of the material being tested. For example, if a material is not likely to cause a change in the permeability of cell membranes, a permeability assay is less apt to determine cytotoxicity in a valid manner. Because MTA is a hydrophilic substance likely to release ionic components, it would be more apt to interfere with intracellular enzyme activities than influence membrane permeabilities (14). Therefore, the MTT assay was chosen for the present study.

Eluates (extracts) of the test materials were used in the present investigation. They offer the advantages of being easily sterilized by filtration, and the ability to examine the effect of materials on cells that are both distant to and in contact with them. Sterilization of the test materials themselves, for direct contact testing, introduces the possibility of changing the properties of the materials. The use of eluates also simulates the immediate postsurgical root-end environment in which toxic elements of the retrofilling material leach into the surrounding fluids in the bony crypt. Eluates can also be made in a series of concentrations to observe a possible dose–response relationship and determine the most ideal concentration for the sensitivity of the cells tested. ISO standard 10993–5:4.2.3.5 calls for the eluates of dental materials for cytotoxicity testing to be prepared so that the ratio of the surface area of the test material to the volume of the extraction vehicle is between 50.0 and 600 mm^2/ml (17). In the present study the surface area to volume ratio used for eluate preparation was 320 mm^2/ml. This was then serially diluted at 1:1, which produced solutions that would be comparable with eluates prepared at ratios of 160 mm^2/ml and 80 mm^2/ml. Conforming to ISO standards allows for a better comparison of results between different studies.

The cell strains used in the present study were human PDL fibroblasts. Previous studies of the cytotoxicity of MTA used established laboratory cell lines (mouse L929 fibroblasts) and human gingival fibroblasts. Established cell lines have the advantage of enhanced reproducibility of results and are recommended by the ISO for preliminary cytotoxicity screening. For specific sensitivity testing to simulate the in vivo situation, primary cell strains derived from living tissues are necessary and are also recommended by the ISO. In this case, human PDL cells were used to simulate the root-end environment. Because of the possible variation between different strains, it is necessary to perform the experiment on primary cell strains derived from different patients and determine the reproducibility of the data. In the present study, two different primary cell strains were used, and ANOVA testing showed the same differences between the materials at a confidence interval of $p < 0.05$ (data not shown).

In the present investigation, MTA proved to be less toxic to human PDL cells than Super-EBA at all concentrations in both the freshly mixed and 24-hr set states. This is in general agreement with previous cytotoxicity studies by Torabinejad et al. (12) and Osorio et al. (18). MTA was also less toxic than amalgam in the freshly mixed state. This is in agreement with Osorio et al., but in contrast to Torabinejad et al. This may be due to differences in experimental procedures. Torabinejad et al. used mouse fibroblasts and an agar overlay method that requires diffusion of toxic components of the material over time. The present study used a primary cell line and eluates of the materials that allow a continuous contact with the cells used.

Within the parameters of an in vitro evaluation, this study supports the use of MTA in the root-end environment.

Dr. Keiser is assistant professor, Department of Biologic and Diagnostic Sciences, and director, Division of Endodontics, University of Tennessee College of Dentistry, Memphis, TN. Mr. Johnson is a third-year student, University of Tennessee College of Dentistry, Memphis, TN. Dr. Tipton is associate professor, Department of Periodontology, University of Tennessee College of Dentistry, Memphis, TN. Address requests for reprints to Dr. Karl Keiser, Department of Biologic and Diagnostic Sciences, University of Tennessee–Memphis, College of Dentistry, 875 Union Avenue, Memphis, TN 38163.

References

1. Pekruhn RB. The incidence of failure following single-visit endodontic therapy. J Endodon 1986;12:68–72.

2. Jokinen MA, Kotilainen R, Poikkeus P, Poikkeus R, Sorkki L. Clinical and radiographic study of pulpectomy and root canal therapy. Scand J Dent Res 1978;86:363–73.

3. Bergenholtz G, Lekholm U, Milthon R. Retreatment of endodontic fillings. Scand J Dent Res 1979;87:217–24.

4. Andreasen JO. Cementum repair after apicoectomy in humans. Acta Odont Scand 1973;31:211–21.

5. Corcoran JF, Sieraski SM, Ellison RL. Osseous healing kinetics after apicoectomy in monkeys. II. A quantitative histologic appraisal. J Endodon 1985;11:269–74.

6. Harrison JW, Jurosky KA. Wound healing in the tissues of the periodontium following periradicular surgery. III. The osseous excisional wound. J Endodon 1991;18:76–81.

7. Andreasen JO, Rud J. Modes of healing histologically after endodontic surgery in 70 cases. Int J Oral Surg 1972;1:148–60.

8. Friedman S. Retrograde approaches in endodontic therapy. Endod Dent Traumatol 1991;7:97–107.

9. Torabinejad M, Watson TF, Pitt Ford TR. Sealing ability of a mineral trioxide aggregate when used as a root end filling material. J Endodon 1993;19:591–5.

10. Torabinejad M, Rastegar AF, Kettering JD, Pitt Ford TR. Bacterial leakage of mineral trioxide aggregate as a root-end filling material. J Endodon 1995;21:109–12.

11. Torabinejad M, Hong CU, Lee SJ, Monsef M, Pitt Ford TR. Investigation of mineral trioxide aggregate for root-end filling in dogs. J Endodon 1995;21:603–8.

12. Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD. Cytotoxicity of four root end filling materials. J Endodon 1995;21:489–92.

13. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. J Dent Res 1988;67:66–70.

14. Schweikl H, Schmalz G. Toxicity parameters for cytotoxicity testing of dental materials in two different mammalian cell lines. Eur J Oral Sci 1996;104:292–9.

15. Wataha JC, Craig RG, Hanks CT. Precision and new methods for testing in vitro alloy cytotoxicity. Dent Mater 1992;8:65–71.

16. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

17. International Standards Organization. 10993-5:4.2.3.5. Biological evaluation of medical devices. Part 5. Tests for cytotoxicity: in vitro methods, 1992.

18. Osorio RM, Hefti A, Vertucci FJ, Shawley AL. Cytotoxicity of endodontic materials. J Endodon 1998;24:91–6.