A review of the methods used to study biocompatibility of Portland cement-derived materials used in dentistry

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Abstract
Advanced restorative dentistry may necessitate the need for surgical intervention to the infected root apex. Once access to the root end is achieved, the root apex is resected and filled with a dental restorative material.

The materials currently in use are not satisfactory due to inadequate biocompatibility and failure to achieve desirable properties in an aqueous environment. With the introduction of a new material, essentially Portland cement used in the building industry, these desirable properties have been achieved. This paper reviews the methods of testing biocompatibility of Portland cement used in dentistry.

Introduction
In restorative dentistry materials are mainly utilized to replace dental tissue lost through dental caries and tooth preparation procedures. When a significant amount of tooth tissue is lost the dental pulp may be adversely affected. This may necessitate advanced conservative procedures involving extirpation of the dental pulp and obliteration of the space with gutta-percha and root canal sealers. Access for these procedures is through the tooth crown. Should this be unsuccessful, surgical endodontics is necessary to allow cleaning and sealing of the root end to prevent further infection.

The main pre-requisites of a material to be used as a root end filling material are biocompatibility and the ability to set in an aqueous environment. Materials of choice for filling the root ends prior to flap repositioning include dental amalgam, glass ionomer cement (GIC), and intermediate restorative material (IRM). None of these materials is the ideal restorative material for the root end of a tooth. Dental amalgam is mercury-based, glass ionomer cements are dimensionally unstable and do not encourage cell growth and IRM is based on eugenol which can be allergenic. The maintenance of a dry field can be problematic during surgery and all these dental cements have to be kept dry until they set.

In the 1990s a new material, mineral trioxide aggregate (MTA) was developed at Loma Linda University as a root-end filling material. The first publication on the use of the material to seal root perforations was published in 1993. It is

Figure 1: Surface morphology of mineral trioxide aggregate showing cell monolayer over the material (x350) (Camilleri et al. 2004).
commercially available as ProRoot MTA (Tulsa Dental Products, Tulsa, OK, USA). The use of MTA as a root-end filling material was identified due to the fact that the material is a hydraulic cement i.e. it sets in the presence of water. A dental cement that sets and develops its properties in the presence of moisture is highly desirable. Mineral trioxide aggregate (MTA) is now used extensively in endodontics. Two commercial forms of MTA are available (ProRoot MTA); namely the grey and the white MTA both with similar chemical and physical properties. MTA is essentially Portland cement (used in the building industry as a binder in concrete) with 4:1 proportions of bismuth oxide added for radiopacity. The material was originally reported to be composed of calcium and phosphate and its biocompatibility was attributed to its similarity to dental hard tissues. However Camilleri and co-workers have shown that MTA is composed primarily of tricalcium and dicalcium silicate, the main constituent elements of Portland cement, which on hydration produce a silicate hydrate gel and calcium hydroxide, not calcium phosphate as claimed by Torabinejad.

As the evidence presented by Torabinejad and co-workers for the biocompatibility of MTA have been shown to be invalid, further studies were necessary to investigate the biocompatibility of MTA.

**Biocompatibility testing**

Biocompatibility testing is performed on materials that are placed in the human body. Human tissue reacts to the material in a variety of ways depending on the material type. These special materials which are able to function in intimate contact with biological fluids or living tissue with minimal adverse reaction or rejection by the body are called biomaterials. The mechanism of tissue attachment (if any) depends on the tissue response to the material surface. Materials can generally be categorised into three classes representing the type of tissue response they elicit: chemically inert, bioreabsorbable, or bioactive.

*In vitro* biocompatibility of a material is usually assessed against two controls: a negative control material, which does not produce a cytotoxic response and a positive control material, which provides a reproducible cytotoxic response. The purpose of the negative control is to demonstrate background response while that of the positive control is to demonstrate appropriate test system response. Tests are performed either on the material itself or on material extracts. Extraction vehicles include culture medium with and without serum, or physiological saline. Materials tested should have at least one flat surface and should be sterilized prior to subjecting it for biocompatibility testing. Biocompatibility of MTA has been tested using the following methods:

1. cell expression and growth
2. animal studies

**Cell expression and growth**

The use of cell cultures in the study of endodontic materials was introduced by Rappaport and co-workers. Cell culture techniques use either primary cells or immortal cell lines, which

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*Figure 2: Surface morphology of cement showing calcium phosphate crystals deposited over the surface of the material (x50 mag.) (Camilleri et al. 2005a)*

*Figure 3: Diffuse calcium carbonate crystals (calcite) deposited over the material after critical point drying x 250 magnification (Camilleri et al. 2004)*

*Figure 4: Large calcium carbonate crystals (aragonite) deposited over the material after critical point drying x 660 magnification (Camilleri et al. 2004)*
are cells derived clonally from normal or malignant cell cultures, usually sarcomas. These cells are easily cultured and they exhibit increased phenotypic stability with serial passages which results in increased reproducibility of results in independently conducted experiments.

Established cell lines have an aneuploid chromosome pattern and thus tend to multiply rapidly with an unlimited life span if appropriately sub-cultured. They are easier to culture and therefore the quality of the cultured cells is more predictable. Aneuploid cells frequently used are L929 mouse fibroblasts, BHK 21 hamster fibroblasts, HeLa human cervical carcinoma epithelial cells and NCTC 2544 human skin epithelial cells.

The SaOS-2 cells represent a highly differentiated cell line capable of inducing bone formation and are thus a model for osteoblastic behaviour. They are more highly differentiated and their growth maintains a consistent cell phenotype. These osteosarcoma cells closely resemble the human osteoblast in its ability to express high levels of bone markers. MG 63 cells are derived from human osteosarcoma cells, however have fewer characteristics of mature osteoblasts.

**Cell type**

MTA and Portland cement show good induction of cell proliferation with the formation of a cell monolayer (Figure 1). Assessment of biocompatibility in vitro using cell culture techniques has been widely used to study the biocompatibility of MTA as a biomaterial. (Table 1). Cell types used varied from immortal cell lines to animal cells and fibroblasts. Cell lines are usually the preferred cell types used for cytological investigations. Primary osteoblasts have however been shown to be more appropriate for testing endodontic materials in cell culture as they are more sensitive and form mineralized nodules when exposed to differentiation media. As cell lines do not behave ostegenically, primary osteoblasts are preferred.

**Evaluation of cell proliferation**

a. **Scanning electron microscopy**

The method preferred for evaluation of cell proliferation was scanning electron microscopy (SEM) followed by enzyme assay. The main problem with the use of the scanning electron microscope in cell culture studies with MTA is the material reaction with the preparation media. Calcium hydroxide, a by-product of calcium silicate hydration, reacts with phosphate buffered solutions producing calcium phosphate crystals over the material surface (Figure 2). Camilleri and co-workers showed that this artifact was responsible for Torabinejad’s conclusion that MTA resembled dental hard tissue. In addition

### Table 1: Cell type, contact time and method of assessment of the various studies conducted on MTA using cells

<table>
<thead>
<tr>
<th>Author and date</th>
<th>Cell type</th>
<th>Contact time/days</th>
<th>Method of assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Torabinejad et al. 1995</td>
<td>Mouse L929</td>
<td>1</td>
<td>Agar overlay</td>
</tr>
<tr>
<td>Torabinejad et al. 1995</td>
<td>Mouse L929</td>
<td>1</td>
<td>Radiochromium release</td>
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<tr>
<td>Koh et al. 1997</td>
<td>MG 63</td>
<td>6</td>
<td>SEM</td>
</tr>
<tr>
<td>Koh et al. 1998</td>
<td>MG 63</td>
<td>1-7</td>
<td>SEM</td>
</tr>
<tr>
<td>Osorio et al. 1998</td>
<td>Gingival fibroblasts, L929</td>
<td>/</td>
<td>MTT, CV assay</td>
</tr>
<tr>
<td>Mitchell et al. 1999</td>
<td>MG 63</td>
<td>2, 4, 7</td>
<td>SEM</td>
</tr>
<tr>
<td>Keiser et al. 2000</td>
<td>PDL fibroblasts</td>
<td>1</td>
<td>MTT assay</td>
</tr>
<tr>
<td>Zhu et al. 2000</td>
<td>HOBs</td>
<td>1</td>
<td>SEM</td>
</tr>
<tr>
<td>Abdullah et al. 2002</td>
<td>SaOS-2</td>
<td>1, 2, 3</td>
<td>SEM</td>
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<tr>
<td>Saiton et al. 2003</td>
<td>Mouse L929</td>
<td>3</td>
<td>SEM</td>
</tr>
<tr>
<td>Haglund et al. 2003</td>
<td>Mouse L929, macrophages</td>
<td>3</td>
<td>SEM</td>
</tr>
<tr>
<td>Huang et al. 2003</td>
<td>U2OS</td>
<td></td>
<td>MTT assay</td>
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<td>Perez et al. 2003</td>
<td>Osteoblasts, MG 63</td>
<td>6, 9, 13</td>
<td>SEM</td>
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<tr>
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<td>PDL, Gingival fibroblasts</td>
<td>4</td>
<td>Cytosolic esterase activity</td>
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<tr>
<td>Camp et al. 2003</td>
<td>Gingival fibroblasts</td>
<td>1, 2, 3</td>
<td>fluorescence</td>
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<td>Asarli and Lobner 2003</td>
<td>Neurons</td>
<td>12-14</td>
<td>enzyme assay</td>
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<td>Balto 2004</td>
<td>PDL fibroblasts</td>
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<td>SEM</td>
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<tr>
<td>Bonson et al. 2004</td>
<td>PDL gingival fibroblasts</td>
<td>15</td>
<td>fluorescence</td>
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<td>SaOS</td>
<td>1, 3</td>
<td>Assay</td>
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<td>Camilleri et al. 2004</td>
<td>SaOS</td>
<td>1, 5, 7</td>
<td>SEM</td>
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<td>Camilleri et al. 2005</td>
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<td>1-7, 1-21</td>
<td>alamarBlue assay</td>
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<td>Sulforhodamine-B assay</td>
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<td>Nakayama et al. 2005</td>
<td>Rat bone marrow cells</td>
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<td>“SEM, TEM”</td>
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<td>Moghaddame-Jafari et al. 2005</td>
<td>Mouse odontoblastic cells</td>
<td>1</td>
<td>Flow cytometry</td>
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critical point drying, an essential step for material preparation prior to viewing under the scanning electron microscope causes cement carbonation (Figures 3, 4).  

b. Enzyme assay

Enzyme assay, the next most preferred method to study cell proliferation on mineral trioxide aggregate, would seem to be more reliable as it avoids material preparation. Enzyme assay measures the metabolic activity of cells grown over the materials under study. This can be done by using alamarBlue™ and methyltetrazolium (MTT) assay in a method outlined in ISO 10993-5. Cell proliferation is determined using a redox indicator that can be used to quantitatively measure proliferation of cells. As the cells grow in culture, their metabolic activity maintains a reducing environment in the surrounding culture medium, whilst growth inhibition produces an oxidized environment. Reduction causes colour change of the alamarBlue™ indicator from non-fluorescent (blue) to fluorescent (red). In addition the cell activity on material elusion can also be measured. This ensures that no toxic substances are leached from the material. The MTT assay is dependent on the intact activity of the mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings. The test involves the conversion of a tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide an insoluble formazan product, which can be quantified by spectrophotometry. Due to the poor knowledge of the chemical constitution of MTA very few studies were published on the material extracts of the material.

c. Cytokine expression

Biocompatibility can also be determined by quantifying the effect of the material on the normal bone physiology. Bone remodeling takes place in alternating phases of resorption and deposition. The first phase involves the recruitment and differentiation of the osteoblast precursors. The second phase is the production and mineralization of the bone matrix by the mature osteoblasts. The cytokines involved in bone formation are divided into cytokines that stimulate bone cell proliferation and those that have an inhibitory effect on the maturing osteoblasts. The former include cytokines such as IL-1, tumour necrosis factor and macrophage-colony stimulating factor. For bone resorption to take place, bone formation must be halted. At this phase, stimulation of osteoblast precursors occurs in preparation for renewed bone formation. Here cytokines stimulate both precursor proliferation and mature osteoblast activity. MTA induced expression of cytokines from bone cells and exhibited good cell attachment. MTA caused an increase in IL-4 and IL-10 levels. Another study showed an increase in Interleukin 6, and 8 with no increase in levels of Interleukin 1α and 1β. Conversely other researchers showed a rise of both IL-1α and 1β together with IL-6 after the cells were in contact with the material for 144 hours. MTA increases osteocalcin levels and also preferentially induces alkaline phosphatase expression and activity in both periodontal ligament and gingival fibroblasts demonstrating an increase in osteoblast mineralizing activity. In one study where no cytokine production was demonstrated, cell lysis and protein denaturing around the MTA was observed.

d. Other methods

The agar overlay method and radiochromium release methods were the least preferred methods to study cell proliferation. The main disadvantage of radiochromium release method is the use of radioactive isotopes.

Animal studies

Recommended methods for the evaluation of dental materials include a preliminary test that provides a general toxicity profile for the potential material and a secondary test that evaluates local toxicity, and a usage test in experimental animals. The effect of the test material on animal tissues can be studied by histological evaluation of the tissues involved. The main problem with these studies is the cost and upkeep of the test animals.

Subcutaneous and intra-osseous implantation

Subcutaneous implantation of the materials in test animals showed MTA initially elicited severe reactions with coagulation necrosis and dystrophic calcification. The reactions however, subsided to mostly moderate with time. Reactions to intraosseous implants of both materials were less intense than with subcutaneous implantation. The main disadvantage with intra-osseous implantation is that the bony cavity created for placement of the material implant is an artificial socket with no resemblance to a tooth suspended in the periodontal ligament. Implantation of MTA in rat connective tissue produced granulations birefringent to polarized light and an irregular structure like a bridge was observed next to the material. In the dentin wall tubules a layer of birefringent granulations was also observed. The tissue reaction to MTA implantation was the most favorable observed in both tibia and mandible of test animals, as in every specimen, it was free of inflammation. In the tibia, MTA was the material most often observed with direct bone apposition.

Histological assessment of peri-radicular tissues

MTA has been used to fill root ends of teeth in experimental animals. The teeth and surrounding bone were then resected and the presence of inflammation around the material used as a marker of material biocompatibility. MTA at the peri-radicular surgical site produced less periradicular inflammation and more fibrous capsules compared with amalgam. In addition, the presence of cementum on the surface of MTA was a frequent finding. The most characteristic tissue reaction to MTA was the presence of connective tissue after the first postoperative week. Inflammation was seen occasionally. Early tissue healing
events after MTA root-end filling were characterized by hard tissue formation, activated progressively from the peripheral root walls along the MTA-soft tissue interface.

Conclusions

Both MTA and Portland cement are bioactive materials. The biocompatibility of the materials had originally been attributed to the chemical similarity to tooth hard tissues namely calcium phosphate. However this has been shown not to be the case. MTA produces calcium hydroxide as a by-product of the hydration reaction. The similarity of action of both MTA and Portland cement to calcium hydroxide had been postulated.

Calcium hydroxide is used extensively in dentistry. When using SEM to study the biocompatibility of dental materials it is imperative to ensure there is no reaction between the material and the reagents used in the experimental procedure. Scanning electron microscopy thus is contraindicated to evaluate cell growth and expression over materials based on Portland cement. Other methods of assessing biocompatibility are thus preferred.

References