

Pulp Capping Materials Alter the Toxicity and Oxidative Stress Induced by Composite Resins in Dental Pulp Culture

 Alison AGNES,  Audi LONG,  Samantha BEST,  Doug LOBNER

ABSTRACT

Objective: Direct pulp capping involves covering exposed pulp to preserve its viability. Calcium hydroxide materials have traditionally been the most commonly used pulp capping compounds; however, they can be toxic, and their success rate in pulp capping is variable. Recently, the compound mineral trioxide aggregate (MTA) has gained wide use for pulp capping. One advantage of MTA is its low toxicity. However, the effects of MTA and calcium hydroxide compounds on the toxicities of other dental materials have not been tested. The aim of this study is to determine whether different pulp capping materials can alter the toxicity of composite restoration materials.

Methods: We used cultured human dental pulp cells to test the toxicities of the calcium hydroxide pulp capping material Dycal and MTA. We then tested the abilities of these compounds to alter the toxicity of the composite materials Durafill and Flow Line and to induce oxidative stress.

Results: As expected, Dycal demonstrated toxicity, while MTA did not. However, when cells were exposed to subtoxic amounts of Dycal or MTA, then exposed to Durafill or Flow Line, changes in the composite materials induced toxicity. Treatment with Dycal had no effect on the toxicity of Durafill, but significantly attenuated the toxicity of Flow Line; meanwhile, MTA significantly enhanced the toxicity of Durafill but had no effect on the toxicity of Flow Line. Early changes in oxidative stress were correlated with later changes in cell death. Statistical calculations were performed using one-way ANOVA followed by the Bonferroni t-test. P-values <0.05 were considered to indicate significant differences.

Conclusion: The results suggest that when choosing a pulp capping material, one factor that should be considered is the impact of that compound on the toxicity of the composite material used for restoration.

Keywords: Composite resins, dental pulp capping, toxicity

HIGHLIGHTS

- Dycal, but not MTA, is toxic to cultured dental pulp cells.
- Dycal protects against Flow Line toxicity, while MTA enhances Durafill toxicity.
- Dycal decreases Flow Line-induced oxidative stress.
- MTA enhances Durafill-induced oxidative stress.
- Pulp capping materials can affect the toxicities of composite materials.

INTRODUCTION

When dental pulp is exposed, direct pulp capping is often performed; in this process, a compound such as calcium hydroxide is placed directly over the exposed pulp to preserve its vitality. An important decision when performing vital pulp therapy is the selection of materials that have limited toxicity to the pulp, thus improving the likelihood of maintaining tissue viability. Calcium hydroxide has long been the

gold standard among direct pulp capping materials for its antibacterial properties and ability to stimulate formation of a reparative dentin barrier (1, 2). However, the success rate of pulp capping using calcium hydroxide compounds is highly variable, and its overall effectiveness has been questioned (3, 4). Due to its high solubility, poor ability to form a seal with the surrounding tooth structure and inherent toxicity to dental pulp cells, the clinical effectiveness of calcium hydroxide is questionable.

A potential alternative to calcium hydroxide compounds is mineral trioxide aggregate (MTA), which was originally used as a root-end-filling material following endodontic therapy. MTA is a complex material composed of various forms of calcium silicate with the addition of bismuth

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From the Department of Biomedical Sciences (A.A., S.B., D.L. ✉doug.lobner@marquette.edu), Marquette University, Milwaukee, USA; Department of Orthodontics (A.L.), Marquette University, Milwaukee, USA.

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oxide for radiopacity. Recently, the popularity of MTA has increased for use in pulpotomy, apical barrier formation and repair of root perforations (5). Because of its high biocompatibility, MTA has also been used as a pulp capping material (6, 7). In addition to its biocompatibility, the advantages of MTA are its good sealing properties and its ability to induce dentin bridge formation (8). However, questions remain concerning the long-term success of its use for pulp capping (9, 10). The potential weaknesses of MTA include its long setting time, poor handling properties, low compressive strength, potential discoloration, lack of known solvent and high cost (11). Thus, while MTA appears to be an improvement over calcium hydroxide compounds, it does not appear to address all the issues involved in pulp capping procedures; thus, its usefulness as a pulp capping agent may be limited.

Teeth requiring direct pulp caps also require restoration, and the choice of restorative material may impact treatment outcomes. Resin-based composites are widely used as restorative materials due to their esthetic properties and ability to bond to tooth structure. Flowable composites are of particular interest; they have been recommended for use as a base layer in deep restorations because they may approximate pulp and may also serve as effective liners, providing a seal for a direct pulp cap (12). Composite materials, however, can be toxic to pulp, resulting in decreased cell viability in culture (13, 14). Because a direct pulp cap will require subsequent placement of an overlying restoration, the combined toxic effects of pulp capping and restorative materials on pulp cells may be more clinically relevant than the effects of the pulp capping material alone. Indeed, a major unstudied variable in clinical studies comparing the success rates of direct pulp capping methods is the selection of the restorative material.

In this study, we measured the viability of dental pulp cells exposed initially to either MTA or Dycal (a fast-setting calcium hydroxide pulp capping material that also contains barium sulfate, urethane dimethacrylate resin, photoinitiator, stabiliser and pigments) followed by exposure to two commonly used composite restorative materials, Durafill and Flow Line, to determine if exposure to these pulp capping materials produced any changes in the toxicities of the restorative materials. Surprisingly, we found that the normally non-toxic MTA potentiated the toxicity of Durafill.

MATERIALS AND METHODS

Materials

Serum was obtained from Atlanta Biologicals (Atlanta, GA, USA). Flow Line and Durafill (Durafill VS) were obtained from Henry Schein Inc. (Melville, NY, USA). MTA (ProRoot MTA) and Dycal (Prisma VLS Dycal) were obtained from Dentsply (Milford, DE, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Subjects and human dental pulp cell cultures

Normal human impacted third molars were collected from adults with informed consent at the Marquette University School of Dentistry Surgical Services Department under an approved protocol by the Marquette University Institutional

Review Board (Protocol Number HR-1004). The tooth surfaces were cleaned and cut around the cementum-enamel junction using sterilised diamond stones to access the pulp chamber. The pulp tissue was separated from the tooth and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 hour at 37°C (15). The cells were plated onto flasks and grown to confluence. The cells were then removed and plated on 24-well plates coated with poly-D-lysine and laminin in Eagle's medium supplemented with 20% fetal calf serum/100 µM L-ascorbic acid 2-phosphate/2 mM L-glutamine/100 units/mL penicillin/100 µg/mL streptomycin; the cells were then incubated at 37°C with 5% CO₂. Experiments were performed on the cultures *in vitro* after seven to nine days on cells which were passed from one to five times; at this time point, the cells formed a confluent layer.

Preparation of dental materials and exposure to cell cultures

Dycal, MTA, Flow Line and Durafill were prepared according to the manufacturer's instructions. Briefly, these materials were dispensed and cured on sterile glass slabs. The composites were polymerised with a visible light curing gun (3M Unitek, St. Paul, MN, USA) for 60 seconds and cut into uniformly sized pieces. Following a 30 minute period, the materials were placed in the cell culture media above the cells.

Cell death assay

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells in the extracellular fluid 24 or 48 hours after the beginning of the insult. The control LDH levels were subtracted from the insult LDH values, and the results were normalised to 100% cell death caused by 20 µM of the calcium ionophore A23187, added 24 hours before the assay. Previous control experiments have shown that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of damaged or destroyed cells (16). The advantages of the LDH release assay for the current study is that it can be performed at multiple time points in the same experiment, which is required for some experiments in the current study and is a measure of true cell death. The commonly used 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) metabolism assay can only be performed at one time point and is a measure of cell activity; this does not always correlate to cell survival, particularly in dividing cells such as those used in the current study (16).

7'-Dichlorofluorescein (DCF) assay of oxidative stress

Oxidative stress was assayed by measuring dichlorofluorescein oxidation using a fluorescent plate reader according to a modification of a previously described method (16, 17). The cultures were exposed to the composite materials for 2 hours, a time point prior to the occurrence of cell death, after which they were exposed to 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) (10 mM). Carboxy-H₂DCFDA is de-esterified within cells to form a free acid which can then be oxidised to fluorescein 2',7'-dichlorofluorescein (DCF). After a 30-minute exposure to carboxy-H₂DCFDA, the cultures were washed 3 times with culture media lacking serum. The fluorescence was then measured using a Fluoroskan Ascent fluorescence plate reader (Thermo Labsystems,

Beverly, MA, USA). The excitation filter was set at 485 nm and the emission filter was set at 538 nm. The background fluorescence (no carboxy-H2DCFDA added) was subtracted, and the results were normalised to the control conditions (carboxy-H2DCFDA added but no dental materials).

Statistical analysis

Statistical calculations were performed using one-way ANOVA followed by the Bonferroni t-test using SigmaStat software (Systat Software Inc.; San Jose, USA). P values <0.05 were considered to indicate significant differences.

RESULTS

Toxicities of dycal and mineral trioxide aggregate

We first determined the relative toxicities of Dycal and MTA. Pieces of Dycal (Figure 1a) or MTA (Figure 1b) of various sizes were freshly prepared as described in the Methods section. Cultured human dental pulp cells were then exposed to different-sized pieces of each material for 48 hours, after which time the extent of cell death was assayed by the LDH release assay. The results show size-dependent toxicity caused by Dycal (Figure 1c), while MTA did not cause toxicity even at weights much larger than those at which Dycal was toxic (Figure 1d).

Effects of dycal and mineral trioxide aggregate on the toxicities of composite materials

While large pieces of Dycal can cause toxicity, we wanted to determine whether smaller, non-toxic pieces of Dycal could alter the toxicities of composite dental materials. Exposure of cultures to pieces of Durafill with standardised sizes (0.0113+0.0002 g) or Flow Line (0.0104+0.0003 g) for 24 hours resulted in cell death rates of approximately 30% to 40% (Figure 2, 3). Exposure of the cultures to a non-toxic-sized piece of Dycal (0.0007+0.0002 g) for 24 hours prior to exposure to the composite materials had no effect on Durafill toxicity (Figure 2a), while the toxicity of Flow Line was actually observed to decrease (Figure 2b). In contrast, exposure of the cultures to a non-toxic sized piece of MTA (0.0049+0.0001 g) for 24 hours prior to exposure to the composites potentiated the toxicity of Durafill (Figure 3a) while having no effect on the toxicity of Flow Line (Figure 3b).

Effects of dycal and mineral trioxide aggregate on oxidative stress induced by composite materials

To determine whether alterations in free radical production played a role in the induction of cell death, we used the DCF assay to assess the free radical levels of cultures after exposure to the dental materials. The cultures were first exposed to

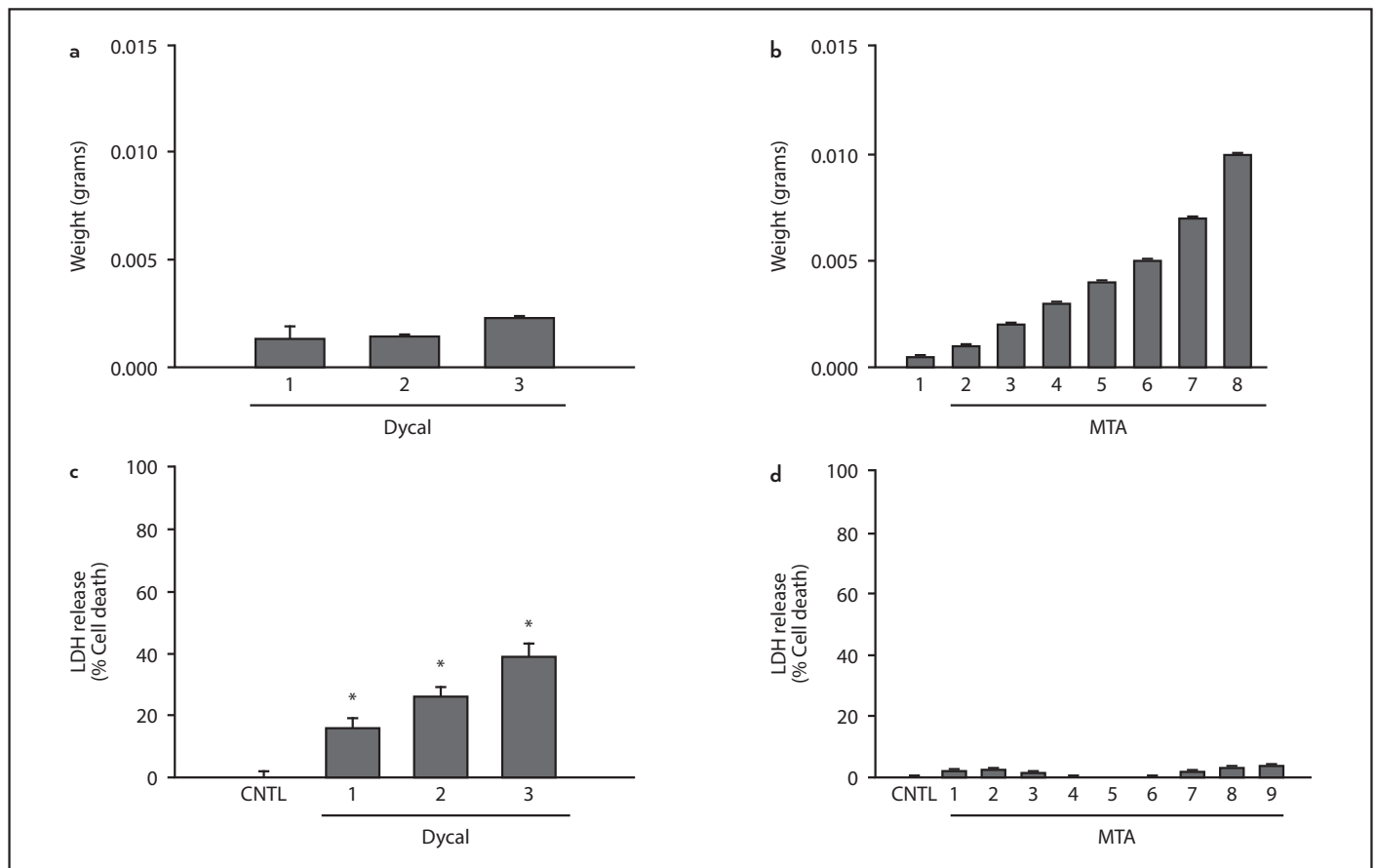


Figure 1. a-d. Dycal causes significant toxicity to dental pulp cells, while MTA does not. Each group of pieces of material with different weights was given a number (Dycal (a) or MTA (b)). Cultured dental pulp cells were exposed for 48 hours to pieces of Dycal (c) or MTA (d) with different weights; after this time, the toxicities were measured by the LDH release assay. The bars show the weights of the materials (mean+SEM, n=8 to 15) (a, b) and the % cell death quantified by release of the cytosolic enzyme LDH (mean+SEM, n=8-15) (c, d)

MTA: mineral trioxide aggregate; LDH: lactate dehydrogenase
 *:indicates a significant difference from the control (P<0.05)

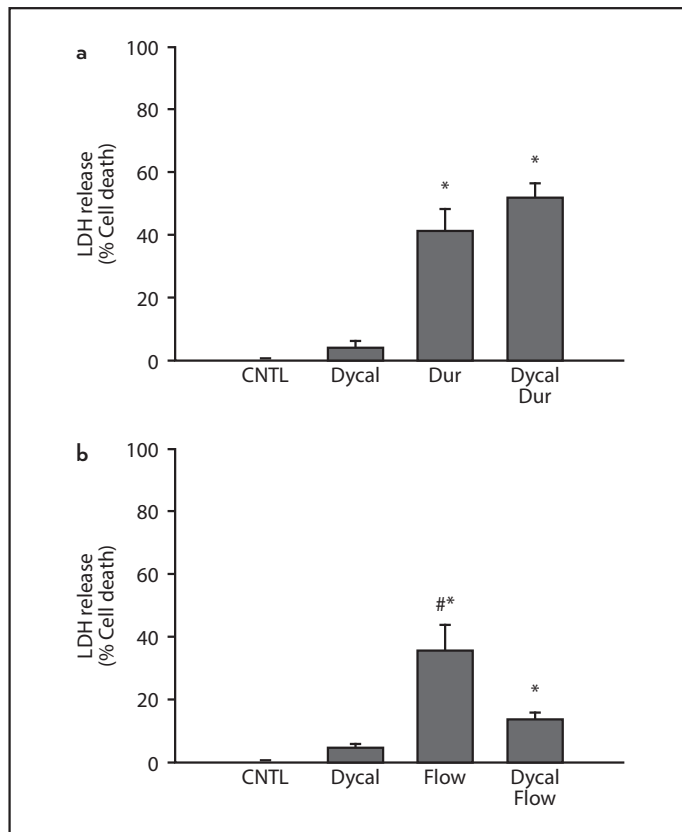


Figure 2. a, b. Exposure to non-toxic-sized pieces of Dycal does not alter Durafill toxicity (a) but attenuates Flow Line toxicity (b). Dycal was added to the cultures for 48 hours, after which Durafill or Flow Line was added for 24 hours. Bars show % cell death, quantified by release of the cytosolic enzyme LDH (mean+SEM, n=8). CNTL (Control); Flow (Flow Line); Dur (Durafill)

LDH: lactate dehydrogenase

*indicates a significant difference from the control ($P < 0.05$)

non-toxic-sized pieces of MTA or Dycal for 24 hours; then, the cultures were exposed to Durafill or Flow Line, and the DCF fluorescence was measured 2 hours later. The free radical levels were measured at this early time point because it precedes the occurrence of cell death; if free radicals induce cell death, their levels should increase before death occurs. Figure 4 and 5 show that Flow Line and Durafill each induced some degree of oxidative stress; in some sets of experiments, this stress was significant (Figure 4a, 5b) and in other sets, it was not (Figure 4b, 5a). Interestingly, pre-treatment with MTA enhanced DCF fluorescence induced by Durafill but had no effect on that induced by Flow Line; meanwhile, pre-treatment with Dycal had no effect on DCF fluorescence induced by Durafill but decreased that induced by Flow Line (Figure 4, 5).

DISCUSSION

This study demonstrates that exposure of dental pulp cells to either Dycal or MTA can alter the toxicities of the flowable composites Durafill and Flow Line. Specifically, Dycal exposure protected pulp cells from the toxicity of Flow Line, while MTA exposure enhanced the toxicity of Durafill. To our knowledge, this is the first study to investigate the combined cytotoxic effects of pulp capping and restorative materials. Because

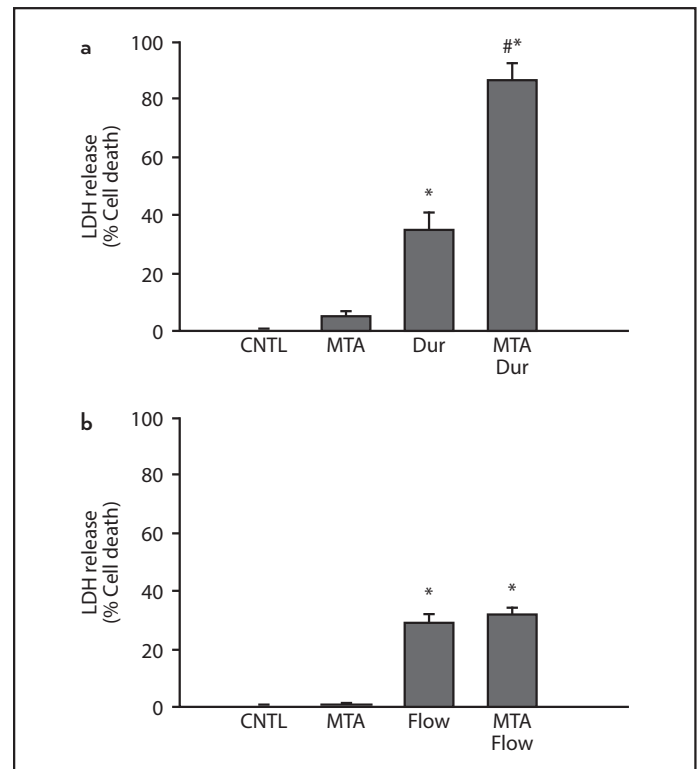


Figure 3. a, b. Exposure to non-toxic-sized pieces of MTA enhances Durafill toxicity (a) but does not alter Flow Line toxicity (b). MTA was added to the cultures for 48 hours, after which Durafill or Flow Line was added for 24 hours. Bars show % cell death, quantified by release of the cytosolic enzyme LDH (mean+SEM, n=8 to 16)

MTA: mineral trioxide aggregate; LDH: lactate dehydrogenase

*indicates a significant difference from the control ($P < 0.05$)

teeth requiring the application of pulp capping material also require subsequent restoration, the combined toxic effects of those materials are of interest and have potential clinical implications. While exposure of dental pulp cells to MTA has been shown to cause little toxicity, the current study shows that MTA can increase the toxicity of restorative materials (18, 19). Conversely, Dycal is known to be toxic to dental pulp cells; however, this study demonstrates its potential protective effects against the toxicity of restorative materials (14, 19, 20). These results raise the possibility that the effectiveness of pulp capping materials may be partially determined by the type of restoration material applied.

The mechanism of these effects appears to be mediated by changes in oxidative stress. However, the toxicities of both Durafill and Flow Line have been shown to be mediated by oxidative stress (14). In the current study, we found a correlation between early changes in oxidative stress measured by DCF fluorescence and cell death measured at a later time point. That is, MTA treatment not only enhanced the oxidative stress induced by Durafill but also enhanced the toxicity of Durafill. Also, Dycal treatment decreased both the oxidative stress and toxicity induced by Flow Line.

The current study is of the greatest interest if the compounds released by composite materials can pass through, or around, a layer of MTA to contact the dental pulp. Numerous studies

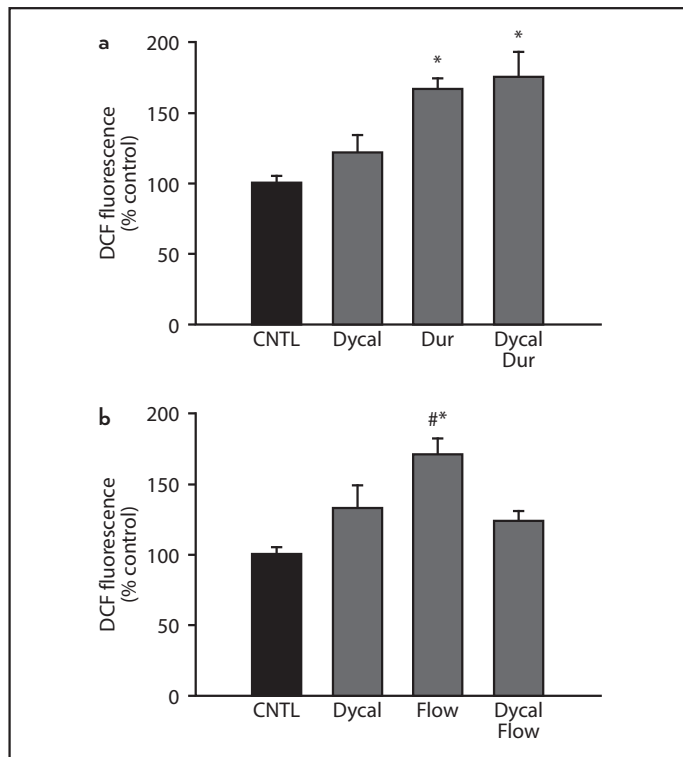


Figure 4. a, b. Exposure to non-toxic-sized pieces of Dycal does not alter Durafill-induced DCF fluorescence (a) but attenuates Flow Line-induced DCF fluorescence (b). Dycal was added to the cultures for 48 hours, after which Durafill or Flow Line was added for 2 hours. Bars show % DCF fluorescence, normalised to the control (mean+SEM, n=7 to 14). CNTL (Control); Flow (Flow Line); Dur (Durafill)
 DCF: dichlorofluorescein
 *indicates a significant difference from the control (P<0.05)

have shown that MTA is very effective in limiting leakage (21). However, these studies were performed under the conditions of the original application of MTA as a root-end filling material. The application and physical condition of materials under root-end filling conditions are different from those during pulp capping. MTA has been shown to develop large pores of the type which allow fluids to penetrate through the material (22, 23). Another concern with MTA is that it is susceptible to washout, which is the tendency of a freshly prepared compound to disintegrate upon contact with fluid (24). Therefore, it is possible for compounds to leak through the MTA layer used for pulp capping.

Because the use of MTA for pulp capping is increasing, an important question is whether the success rate of MTA is greater than that of calcium hydroxide compounds. In a review of the efficacy of MTA for endodontic therapy, the authors concluded that at that time there was 'no evidence that MTA was better than present materials and techniques as a pulpotomy medicament' (25). This does not necessarily indicate that MTA is not more effective, but that the studies performed at that time lacked the power to enable conclusions regarding its use. Two recent large, long-term trials have directly compared the success rate of MTA vs that of a calcium hydroxide compound in direct pulp capping (26, 27). The results of the studies were very similar, with MTA success rates of 80.3% and 80.5% and calcium hydroxide compound success rates of 68.5% and 59%. The study by Hilton et al.

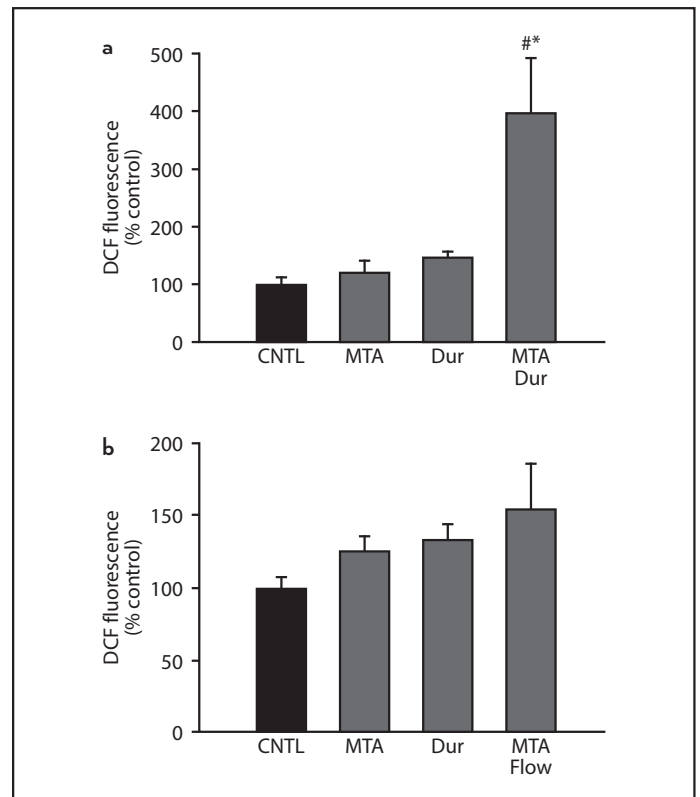


Figure 5. a, b. Exposure to non-toxic-sized pieces of MTA enhances Durafill-induced DCF fluorescence (a) but does not alter Flow Line-induced fluorescence (b). MTA was added to the cultures for 48 hours, after which Durafill or Flow Line was added for 2 hours. Bars show % DCF fluorescence normalised to the control (mean+SEM, n=7 to 16)
 MTA: mineral trioxide aggregate; DCF: dichlorofluorescein
 *indicates a significant difference from the control (P<0.05)

(26) used a hard-set form of calcium hydroxide (Life), while the study by Mente et al. (27) used a non-setting calcium hydroxide paste (Hypocal SN). The results suggest that MTA is somewhat more effective than calcium hydroxide compounds for pulp capping; however, even under these controlled conditions, it was not always effective. A limitation of these studies is that the type of restoration material used was not described. Also, some factors may limit the practical use of MTA for pulp capping. The disadvantages of MTA include high cost and slow setting time as well as difficulty of handling (11, 28).

Many studies have shown that MTA is non-toxic (21). However, MTA is clearly biologically active. In fact, this is one of its benefits, as MTA can induce dental pulp cell differentiation and stimulate the production of reparative dentin (8, 29). At least some of the biological effects of MTA are likely due to the fact that it releases calcium hydroxide (30). Therefore, while MTA has not been shown to be toxic like calcium hydroxide-based compounds, it clearly alters the function of dental pulp cells and could therefore alter their susceptibility to the toxicities of other materials.

CONCLUSION

There are important limitations to *in vitro* studies such as those presented here. For example, when the pulp capping and restorative materials were introduced to the pulp cells in cul-

ture, they were not placed in consecutively applied layers overlying the pulp cells as they would be in a clinical scenario. However, this study does suggest that the restoration material used following pulp capping may be important and at a minimum should be tracked when performing pulp capping studies.

Disclosures

Ethical Approval: Ethics committee approval was received for this study from the Marquette University Institutional Review Board (Protocol Number: HR-1004).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: No conflict of interest was declared by the authors.

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