Changes in Composite Toxicity Following Exposure to Pulp Capping Materials

Audra Long
Marquette University

CHANGES IN COMPOSITE TOXICITY FOLLOWING EXPOSURE TO PULP CAPPING MATERIALS

by

Audra M. Long, DDS

A Thesis Submitted to the Faculty of the Graduate School, Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Master of Science

Milwaukee, Wisconsin

August 2015

ABSTRACT
CHANGES IN COMPOSITE TOXICITY FOLLOWING
EXPOSURE TO PULP CAPPING MATERIALS

Audra M. Long, DDS

Marquette University, 2015

Introduction: Direct pulp capping involves placing a material over the exposed pulp in order to maintain its vitality. For decades, calcium hydroxide (CH) has served as the gold standard for this purpose, but its toxicity to the pulp may negatively impact treatment outcomes. Mineral Trioxide Aggregate (MTA) has become a popular alternative based partly on its excellent biocompatibility. Pulp-capped teeth are often restored with highly toxic composite materials, but the pulp capping material's ability to alter these toxic effects has never been investigated. The purpose of this *in vitro* study is to determine the effects of Dycal, a CH-based cement, and ProRoot MTA on the toxicities of two popular restorative composites, Flow Line and Durafill VS.

Materials and Methods: Human dental pulp cells were cultured and exposed to Dycal or MTA for 48 hours. Dycal and MTA were then removed and either Flow Line or Durafill VS was added to cell cultures for 24 hours. Toxicity was determined using the LDH release assay before and after the addition of the composite material.

Results: Dycal demonstrated a high level of toxicity that correlated with the amount of material placed in cell culture. MTA was nontoxic even in amounts at which Dycal was highly toxic. Exposure of pulp cells to Dycal resulted in decreased toxicity of Durafill VS and had no effect on Flow Line toxicity. MTA exposure resulted in enhanced Flow Line toxicity and had no effect on the toxicity of Durafill VS.

Conclusions: These results show that calcium hydroxide and MTA are capable of altering the toxicity of composite restorative materials. MTA may enhance the toxicity of some composites, while Dycal may have an inhibitory effect. More studies are needed to determine the clinical significance of these effects.

ACKNOWLEDGMENTS

Audra M. Long, DDS

I would first like to express sincere gratitude to my advisor, Dr. Doug Lobner, who has supported me throughout this work and taught me a great deal about conducting meaningful research. I would also like to thank Dr. Sheila Stover for sharing her time and clinical expertise with me in addition to offering constructive suggestions regarding the content of this written work. My committee would not be complete without the council of Dr. T. Gerard Bradley and Dr. Dawei Liu who not only supported this research and saw to its timely completion, but are also largely responsible for the excellent orthodontic education I have received at Marquette. I am eternally grateful to my patient and loving husband, Micah, who spent hours recovering this document from the deep recesses of my hard drive when Microsoft Word crashed and deleted it in its entirety.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Direct pulp capping is a form of vital pulp therapy wherein a material is placed directly over the exposed pulp with the goal of preserving its vitality. Success with this procedure is dictated by the formation of a reparative dentin bridge with minimal communication between the capping material and the pulp (1). Clinical studies have demonstrated favorable long-term success rates under appropriate conditions (2-5) However, the toxicities of dental materials used for the direct pulp cap and the restoration that covers it are concerning. Even in the absence of bacteria, these materials can cause severe inflammation and necrosis when placed in direct contact with the pulp leading to failure of the pulp cap and the need for root canal treatment (6). Cytotoxicity of dental materials may pose insurmountable challenges to the pulp's defense mechanisms, especially when there is pre-existing inflammation due to trauma, caries, bacterial contamination, or iatrogenic damage (7). Therefore, dental materials placed in close proximity to the pulp should ideally possess excellent biologic properties and encourage healing if they are to be predictably successful in maintaining pulpal vitality.

For decades, calcium hydroxide (CH) has served as the material of choice for direct pulp capping. Its high alkalinity creates an environment that promotes therapeutic benefits such as mineralization of hard tissue and inhibition of bacterial growth. However, the alkalinity is also extremely toxic to pulp cells. When in direct contact with the pulp, CH produces inflammatory changes and a superficial layer of coagulative necrosis, leaving it up to the subadjacent pulp to generate healing and form a hard tissue

barrier (8). Therefore, despite CH's ability to preserve pulpal vitality in the face of injury, it lacks inherent biocompatibility, and its damaging effects may result in failure of the procedure (6, 9).

Mineral Trioxide Aggregate (MTA) is a newer material that has gained favor among clinicians for numerous endodontic applications, including direct pulp capping. Interestingly, CH is formed during MTA's setting reaction, which imparts MTA with antibacterial and regenerative properties. For this reason, MTA and CH are thought to share a similar mechanism of action (10). However, studies show less inflammation, better dentin bridging, and minimal cytotoxicity with MTA (11-15). These findings imply an MTA-specific mechanism, which studies have strongly suggested involves the formation of hydroxyapatite upon exposure of MTA to physiologic solutions (10, 16). MTA may also have bioinductive capabilities, stimulating the release of morphogenetic proteins and growth factors such as BMP-2 and TGF-β1 (17).

In addition to reparative and antibacterial benefits, the pulp cap also serves as a physical barrier that protects the pulp from the external environment while new hard tissue is forming. Porosity, solubility, and poor sealing properties of the pulp capping material, however, may limit its ability to shield the pulp from the harmful effects of bacteria or toxic compounds leached from overlying restorative materials. Today, resin-based composites are popular restorative materials due to their esthetic properties and ability to chemically bond to tooth structure. However, the methacrylate monomers contained in composite materials are highly cytotoxic and may interfere with the immune response of the pulp, weakening its ability to resist bacterial challenge (7, 18, 19). Composites have been shown to cause chronic pulpal inflammation and prevent reparative dentin formation when applied directly to pulp exposures (6, 20).

No studies to date have investigated the ability of pulp capping materials to alter the toxicity of composites. The purpose of this study is to determine the effects of two pulp capping materials: Dycal, a CH-based cement, and ProRoot MTA on the toxicities of Flow Line and Durafill VS, two popular composite restorative materials.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Dental pulp function and cellular composition

The dental pulp serves to form and nourish the dentin as well as provide a source of innervation and protection from injury. Anatomically, it consists of loose connective tissue, nerve endings and small blood vessels. The cellular composition is complex and changes in the presence of inflammation.

Chapter 2 of the textbook *Endodonti*cs by Pashley, Walton and Slavkin (2002) provides a thorough summary of the cellular composition of normal, healthy pulp tissue. In short:

1. *Fibroblasts.* These cells comprise the majority of cells within the pulp and are responsible for the formation and degradation of collagen and ground substance. Unlike typical connective tissue fibroblasts, however, many pulpal fibroblasts are capable of forming hard tissue.
2. *Odontoblasts.* Located at the periphery of the pulp, in contact with the dentinal interface, these are the main cells responsible for the formation of dentin.
3. *Dental Pulp Stem Cells (DPSCs)*. These multipotent mesenchymal cells retain the ability to differentiate into a number of mature cell types throughout life. They are responsible for reparative dentin formation beneath pulp capping materials by differentiating into odontoblasts for this purpose.

4. *Dendritic cells.* As the most prominent immune cell in the healthy dental pulp, they are responsible for activating the immune response through recognition and presentation of foreign antigen.
5. *Histiocytes and Macrophages.* These are phagocytes that can be found within healthy pulp tissue. They are responsible for removing bacteria, foreign material and dead cells. (21).

With cellular injury or death, inflammatory cells rapidly migrate to the pulp from nearby capillaries and venules. Neutrophils are the most common leukocyte in pulpal inflammation. They function to clear sources of inflammation via phagocytosis, repair tissue damage, and amplify the immune response. This amplification, however, can exacerbate injury, leading to larger areas of inflammation. The presence of other inflammatory cell types including lymphocytes, plasma cells and mast cells signifies the presence of a chronic inflammatory process. (21).

The Direct Pulp Cap

A. Objectives of Direct Pulp Capping

The principle goal of direct pulp capping is to maintain pulpal vitality by stimulating reparative dentin formation. Reparative dentin provides a natural source of pulpal protection from bacteria and dental materials (22). Pulp capping materials are therefore evaluated heavily on their ability to regenerate a hard tissue barrier. A successful direct pulp cap can eliminate the need for root canal treatment, thus avoiding a more invasive, expensive, and time-consuming intervention. Vital teeth show higher rates

of long-term survivability than endodontically treated teeth, particularly for molars (23). Therefore, preservation of vital tooth structure is favorable and, when indicated, direct pulping capping can help attain this goal.

B. Prognosis & Success Rates of Direct Pulp Capping

Direct pulp caps are deemed successful when there is formation of a hard tissue bridge with minimal communication between the capping material and the pulp (1). Case selection is key, as direct pulp capping is not indicated for all pulp exposures. Rather, the decision to place a direct pulp cap should be based on the pulpal and periradicular diagnoses and the conditions under which the exposure occurred (24).

The state of pulpal health and degree of inflammation at the time of exposure dictates the ability for healing to occur and the direct pulp cap to be successful (21, 24). Proper pulpal diagnosis is essential, and vitality testing should always precede treatment of any tooth where there is evidence or suspicion of caries approximating the pulp. Direct pulp caps can be considered for teeth with viable and healthy or reversibly inflamed pulp status and are contraindicated in permanent teeth with closed apices and evidence of irreversible pulpitis or pulpal necrosis (25). Determining whether a pulp is reversibly vs. irreversibly inflamed using vitality tests and patient-reported symptoms can be difficult and inaccurate (26-28). The ability to control pulpal hemorrhage at the time of exposure may be a more reliable indicator of inflammatory status (4). If uncontrollable bleeding exists in a permanent tooth with a closed apex, irreversible pulpitis is the likely diagnosis, and root canal therapy is the appropriate treatment.

The presence of bacteria in the pulp is the greatest cause of direct pulp cap failures (29). This is best judged clinically by whether the exposure occurred during caries removal (carious exposure) vs. cavity preparation on noncarious tooth structure (mechanical exposure). Most practice guidelines including those published by the American Association of Endodontists advise that direct pulp caps are indicated only for mechanical exposures (30). However, a recent systematic review found direct pulp caps placed on carious exposures to have high long-term success rates ranging from 87.5% to 95.4% (3). This is comparable to the 70-98% success rates seen with noncarious mechanical exposures (2).

Other important factors to consider are degree of isolation at the time of exposure and the ability to provide a well-sealed definitive restoration in a timely manner. Bacterial contamination from saliva during cavity preparation or as a result of microleakage beneath the restoration and pulp cap will reduce success rates considerably (7, 31). For this reason it is imperative that a rubber dam be used during any restorative procedure wherein pulp exposure is a suspected outcome, and care should be taken to optimize the marginal seal of the final restoration (32). To increase the likelihood of long-term success, the permanent restoration should be placed within 2 days of the direct pulp cap (5).

Dental materials used for pulp capping and restorative procedures have been shown to elicit cytotoxic and immunosuppressive effects on the pulp (6). The closer the material is to the pulp, the greater effect (33). Therefore, choosing materials that limit damage and promote healing are optimal for situations where direct contact between pulp and dental material is unavoidable.

Direct Pulp Capping Materials and Toxicity

A. Calcium hydroxide (CH)

CH was introduced to dentistry in 1920 by Hermann (34). Today it has a number of clinical applications and is considered the gold standard among pulp capping materials. CH's main activity comes from the dissociation of calcium (Ca^{2+}) and hydroxyl (OH^-) ions when CH is in contact with aqueous fluids. The pH values of most current CH-based cements such as Dycal range from 10-12 (9). The alkalinity stimulates reparative dentin formation and kills bacteria, but is also extremely toxic to pulp cells (1). When in direct contact with the pulp, CH produces a superficial layer of coagulative necrosis up to 2mm in depth as well as inflammatory changes in deeper tissue (8).

Reparative dentin formation is a result of the pulp's defense mechanisms against CH's irritating effects (15). The exact mechanism of induced hard tissue formation is poorly understood. Not only does the dentin barrier serve to protect the pulp from future injury but is also a sign of biological recovery (35). Several in vitro and animal studies have detected tunnel defects in dentin bridges that form in response to CH (36). Such disruptions in the dentin barrier could compromise its protective benefits by serving as conduits for microleakage (35). However, Hilton reported in a 2009 review that tunnel defects related to CH were a less common finding in human studies(37).

Another advantage of CH is its ability to inhibit bacterial growth. This effect is produced by the hydroxyl ions released from CH in an aqueous environment (38). Hydroxyl ions are highly oxidant free radicals, with extreme reactivity capable of causing bacterial cell death (38).

Despite its advantages, CH is highly soluble and lacks inherent sealing capabilities. These properties can create opportunities for bacterial contamination (39). Therefore, CH pulp caps require placement of an overlying hard setting liner such as a glass ionomer (GI) or composite-based cement to provide an adequate seal and reduce microleakage.

Studies have identified two undesirable consequences of CH pulp caps. First, CH can produce a persistent stimulating effect on dentin formation, leading to pulpal obliteration (8, 24). If root canal treatment is needed in the future, the hypercalcification can make this procedure difficult if not impossible. Another potential adverse effect of direct pulp caps with CH is chronic inflammation, which can eventually lead to internal resorption (8, 24).

B. Mineral Trioxide Aggregate (MTA)

Mineral Trioxide Aggregate (MTA) was originally developed in 1993 by Torabinejad et al. as a root end filling material and is now a popular choice among clinicians for direct pulp capping as well. MTA is a refined Portland cement with bismuth oxide added for radiopacity. Portland cement is the main ingredient in mortar and concrete. It contains calcium silicate, tricalcium silicate, tricalcium aluminate, gypsum, and tetracalcium aluminoferrite (10). MTA exists as a powder that is mixed with water in a 3:1 powder/liquid ratio to form a silicate hydrate gel that hardens as it sets. Calcium hydroxide is also formed during this hydration reaction, resulting in the high alkalinity of MTA. Its pH increases from 10.2 during manipulation to 12.5 after setting (40).

MTA has a number of properties that are desirable in a pulp capping material. First, it is recognized as one of the most biocompatible dental materials available (41). In

fact, its cytotoxicity has been likened to that of titanium alloy, which is chemically inert (41, 42). It induces limited tissue necrosis and inflammation in vivo and is also capable of inducing hard tissue formation at a faster rate and of greater thickness and quality than CH-based materials (13, 43-45). MTA is also able to form an excellent seal with tooth structure that protects against bacterial leakage (41, 46, 47). This is a major advantage over CH-based materials, as bacterial contamination is the greatest threat to the pulp's healing capacity (48). Finally, MTA has an antibacterial effect, although it is less robust than that of CH (15).

Despite its many advantages, MTA has some important drawbacks that may limit its effectiveness. Perhaps its greatest drawback is a prolonged setting time of up to 4 hours (49, 50). For this reason, it is ideal to place a moist cotton pellet and temporary restoration over the unset MTA to allow for complete setting and avoided disturbance before the definitive restoration is placed, usually at a consecutive visit. To avoid the need for an additional visit, another acceptable approach is to place a hard-set lining material over the unset MTA, followed immediately by the definitive restoration (37, 50). Another shortcoming of MTA is its porosity, which may limit its ability to shield the pulp from bacteria and other irritants (51). The porosity increases with the amount of water added, incorporation of air bubbles when mixing, and the acidity of the local environment (47).

Like CH, MTA's mechanism of action lacks detailed understanding. Many investigators believe that because CH is formed during the setting reaction, their mechanisms are similar or identical (43, 52). However, MTA's enhanced biologic properties suggest activity that is unique to MTA. While only a few studies have investigated the specific quality of MTA that provides its favorable biocompatibility, there is strong evidence that it is due to its ability to form hydroxyapatite in physiologic solutions (10). MTA has also demonstrated bioinductive capabilities, promoting the formation of morphogenetic proteins and growth factors such as BMP-2 and TGF-β1(17).

Clinical Success Rates: CaOH vs. MTA

A recent Cochrane Review found a lack of evidence as to the most effective pulp capping material(53). MTA was not considered in this review, as no long-term randomized controlled trials were available for inclusion.

A 2009 systematic review comparing short-term treatment outcomes of CH vs. MTA direct pulp caps concluded that due to a lack of high quality studies on MTA, CH should still be considered the gold standard for direct pulp capping (37). However, in 2014, Mente et al. published the largest controlled clinical trial to date comparing long-term outcomes of direct pulp caps performed with MTA and CH (5). The authors found direct pulp caps performed with CH had a failure rate 2.5 times that of MTA and concluded that MTA was a superior material.

While these results seem promising for MTA, the results of another long-term clinical trial conducted in 2013 by Hilton et al. were less convincing (54). This study was conducted in a practice-based research network where adherence to study protocol could

not easily be monitored. While the authors reported higher failure rates for direct pulp caps carried out with CH (31.5%) vs. MTA (19.7%), exclusion of one practice with an inordinate number of failures from the data pool reduced the difference in failure rate between the two materials to statistically insignificant values. Interestingly, the study by Mente et al. (2014) required rubber dam isolation as part of the treatment intervention while the Hilton et al. study (2013) reported rubber dam use in only 19% of cases on average (15% of CH pulp caps and 22% of MTA pulp caps).

While there is sufficient evidence to support MTA's safety and efficacy as a direct pulp capping material, additional studies of high quality that clearly indicate superior outcomes with MTA are needed before it can officially replace CH as the gold standard. More studies of MTA applied to carious exposures are especially needed in order to evaluate its full potential in clinically relevant situations.

Composite Restorative Materials

The key to the long-term success with direct pulp capping is a well-sealed restoration (37). Resin-based composites are a popular choice of restorative material due to their esthetic properties and ability to chemically bond to tooth structure. They consist of a resin matrix usually containing bis-GMA in addition to inorganic glass fillers and silane coupling agents (55).

A major disadvantage to the use of composite materials is their toxicity to the pulp. Several studies have confirmed the cytoxicities of various composite restorative materials (7, 12, 56). The mechanism appears to involve the impairment of mitochondrial function, producing irreversible effects on cellular metabolism (56, 57). In vivo studies

have shown composite restorations to be associated with pulpal irritation and necrosis (6). The organic monomers contained in the resin phase of composite materials such as bisphenol A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA), and 2-hydroxyethyl methacrylate (HEMA) are thought to be largely responsible for these toxic effects (6). These monomers are leached from composites that have not reached complete conversion and can diffuse through dentin tubules as well as porous or poorly sealed pulp caps to reach the pulp (6).

The toxicity of composite materials may interfere with the regenerative goals of direct pulp capping. Studies point to the ability of composites to prevent reparative dentin formation following pulp exposure by inhibiting odontoblast differentiation (18). Lack of a hard tissue barrier greatly reduces the pulp's ability to combat bacterial and chemical irritants and over the long-term will almost certainly result in vitality loss. Therefore, placing composite materials in close proximity to pulp may negatively influence outcomes of vital pulp therapy.

In spite of this, composite materials are commonly used for deep restorations including those that require a direct pulp cap. If the definitive restoration is to be placed immediately after an MTA pulp cap, a hard setting composite-based liner is often used to protect the unset MTA from disturbance during the restorative process. Composite liners are also recommended for placement over CH pulp caps for the purpose of providing an adequate seal, which CH-based cements inherently lack. A composite restoration is often placed on top of the lining material.

A review of the literature produced no studies that have investigated the ability of pulp capping materials to protect against or alter the toxicity of composites. This information is greatly relevant to the clinical picture, as pulp capping materials and composites are frequently used together in practice.

CHAPTER III

MATERIALS AND METHODS

Materials

Serum was obtained from Atlanta Biologicals (Atlanta, GA, USA). Flow Line and Durafill VS were obtained from Henry Schein Inc. (Melville, NY, USA). MTA and Dycal were obtained from Densply (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 at the Marquette University School of Dentistry Surgical Services Department. After the external surfaces were cleaned, the teeth were sectioned and pulp tissue was removed using sterile hand instruments in a cell culture hood. The pulp tissue was digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 hour at 37ºC (58, 59). The cells were plated onto 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, and then incubated at 37ºC with 5% CO_2. Experiments were performed on cultures 7-9 days in vitro.

Preparation of dental materials and exposure to cell cultures

Dycal, ProRoot MTA, Flow Line, and Durafill VS were dispensed onto a sterile glass slab and prepared according to the manufacturer's instructions. ProRoot MTA powder was gradually added to the liquid provided in the ProRoot micro-dose ampoule (3:1 powder:liquid ratio) and mixed for 1 minute to ensure adequate hydration. Equal volumes of Dycal base and catalyst pastes were mixed until a uniform color was achieved. Composites were polymerized with a visible light curing unit from 3M Unitek for 60 seconds and cut into uniformly sized pieces. Testing was conducted 30 minutes after initial mixing or light curing.

Cell death assay

Cell death was assessed in mixed cultures by the measurement of lactate dehydrogenase (LDH) released from damaged or destroyed cells 24 or 48 hours after the beginning of the insult. Control LDH levels were subtracted from insult LDH values and results normalized to 100% cell death caused by 20 μM of the calcium ionophore A23187 added 24 hours before the assay. Control experiments have shown previously that the efflux of LDH occurring from either necrotic or apoptotic cells is proportional to the number of cells damaged or destroyed (60, 61). Advantages of the LDH assay for the current study are its ability to be performed at multiple time points and act as a measure of true cell death. The MTT metabolism assay commonly used in toxicity studies can only be performed at one time point and is a measure of cell activity, which does not always correlate to cell survival (61).

Testing

I. Dose-Dependent Toxicity of Dycal and ProRoot MTA

Variously weighted pieces of freshly prepared Dycal and MTA were placed on top of cultured human pulp cells for 48 hours. The LDH release assay was performed to assess cell death.

II. Toxicity of Composites

Cultured pulp cells were exposed to standardized sized pieces of Durafill VS (0.0113 $\pm$ .0002 g) and Flow Line (0.0104 $\pm$ .0003 g) for 24 hours. The LDH release assay was used to assess the toxicity of the composite materials.

III. Toxicity of Composites Following Exposure to Dycal or ProRoot MTA

Nontoxic, uniform sizes of Dycal (0.0007 $\pm$ .0002 g) or MTA (0.0049 $\pm$ .0001 g) were placed on top of cultured pulp cells. After 24 hours of exposure, Dycal and MTA were removed and standardized sized pieces of Durafill VS (0.0113 $\pm$ .0002 g) or Flow Line (0.0104 $\pm$ .0003 g) were added to the cell cultures for 24 hours. The LDH release assay was performed once again to assess cytotoxicity.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by the Bonferroni post-hoc comparison. P-value <0.05 was considered to indicate significant difference.

CHAPTER IV

RESULTS

Dose-Dependent Toxicity of Dycal vs. MTA

First, we set out to determine if there is a relationship between the toxicity of Dycal or MTA and the amount of material placed in cell culture. Various weighted pieces of Dycal (Fig. 1) and MTA (Fig. 2) were prepared and placed on top of cultured human pulp cells for 48 hours. The LDH release assay was performed to assess cell death. Dycal demonstrated a significantly higher degree of toxicity than MTA. Dycal's toxicity increased accordingly with greater amounts of material (Fig. 3), while MTA was relatively nontoxic even in amounts much larger (heavier?) than those at which Dycal was highly toxic (Fig. 4). These findings are consistent with those of previous studies comparing toxicities of CH- and MTA-based materials (13-15, 62).

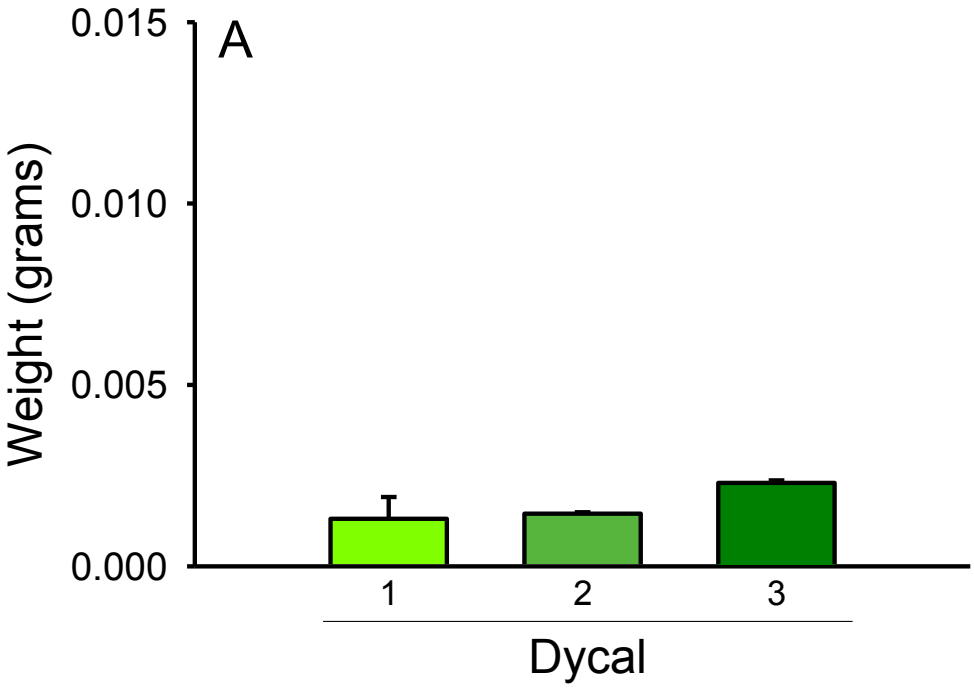

Figure 1. Weights of Dycal placed in cell cultures.

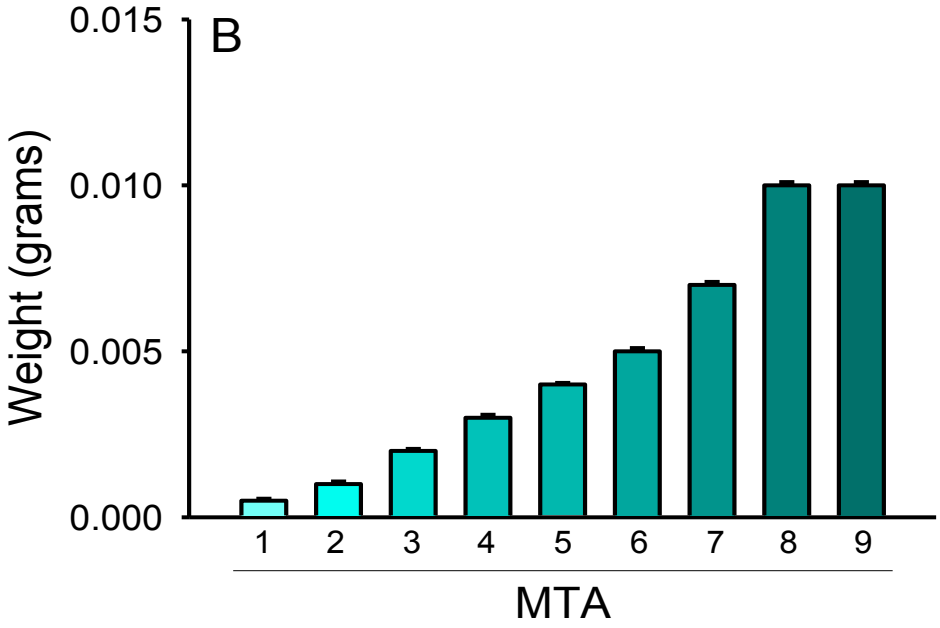

Figure 2. Weights of ProRoot MTA placed in cell cultures.

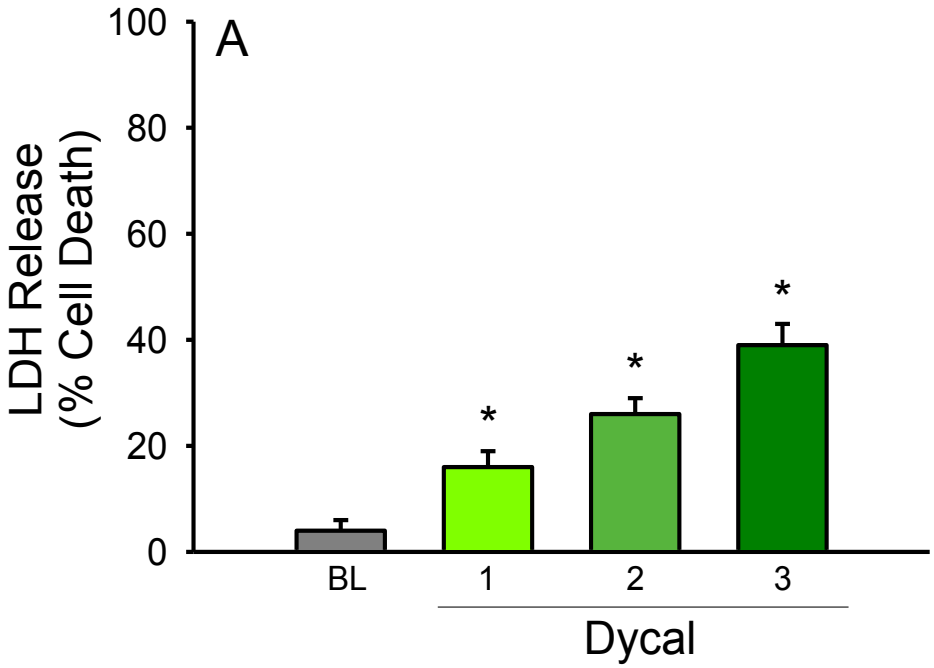

Figure 3. Dose-dependent Toxicity of Dycal. The toxicity of Dycal was positively correlated with the amount of material placed in cell culture. *** denotes significant difference from control (BL) ($p<0.05$).**

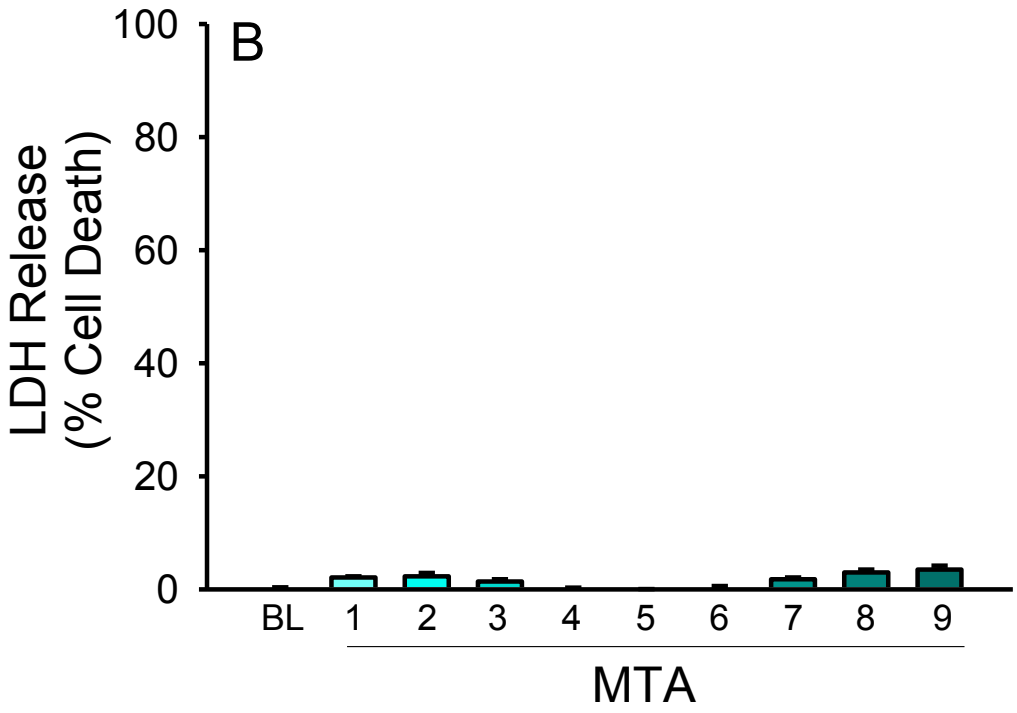

Figure 4. Dose-dependent toxicity of ProRoot MTA. ProRoot MTA was relatively nontoxic, even at amounts greater than those at which Dycal was highly toxic ($p<0.05$).

Toxicity of composites following Dycal or MTA exposure

We next set out to test the toxicity of composites to human pulp cells following prior exposure to either Dycal or MTA. To rule out incidence of cell death resultant of exposure to Dycal alone, we used a nontoxic-sized piece of Dycal, as determined in the first part of the experiment. When cultured pulp cells were exposed to standardized sized Durafill VS (0.0113 $\pm$ .0002 g) and Flow Line (0.0104 $\pm$ .0003 g) for 24 hours, the result was approximately 30-40% cell death. When nontoxic, uniform sizes of Dycal (0.0007 $\pm$ .0002 g) or MTA (0.0049$\pm$ .0001 g) were placed on top of cultured pulp cells for 24 hours prior to their exposure to the composite materials, the results were different. Exposure to Dycal had no effect on Durafill toxicity but decreased Flow Line toxicity (Fig 5). Exposure to MTA enhanced Durafill toxicity and had no effect on the toxicity of Flow Line (Fig 6).

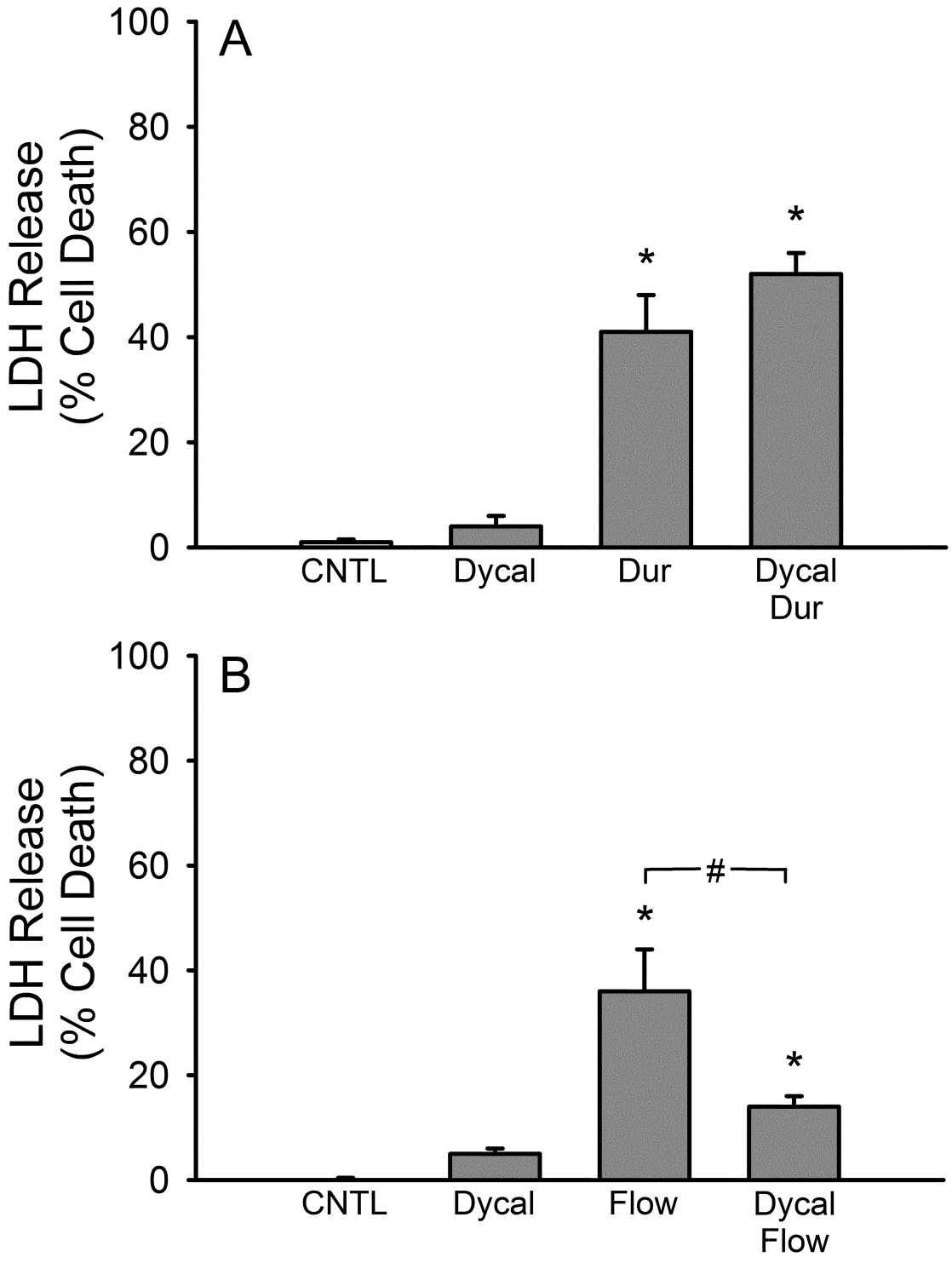

Figure 5. Effect of Dycal on toxicities of Durafill VS and Flow Line. Dycal did not significantly alter the toxicity of Durafill VS, while it significantly decreased the toxicity of Flow Line ($p<0.05$). *denotes significant difference from control; # denotes significant difference between samples.

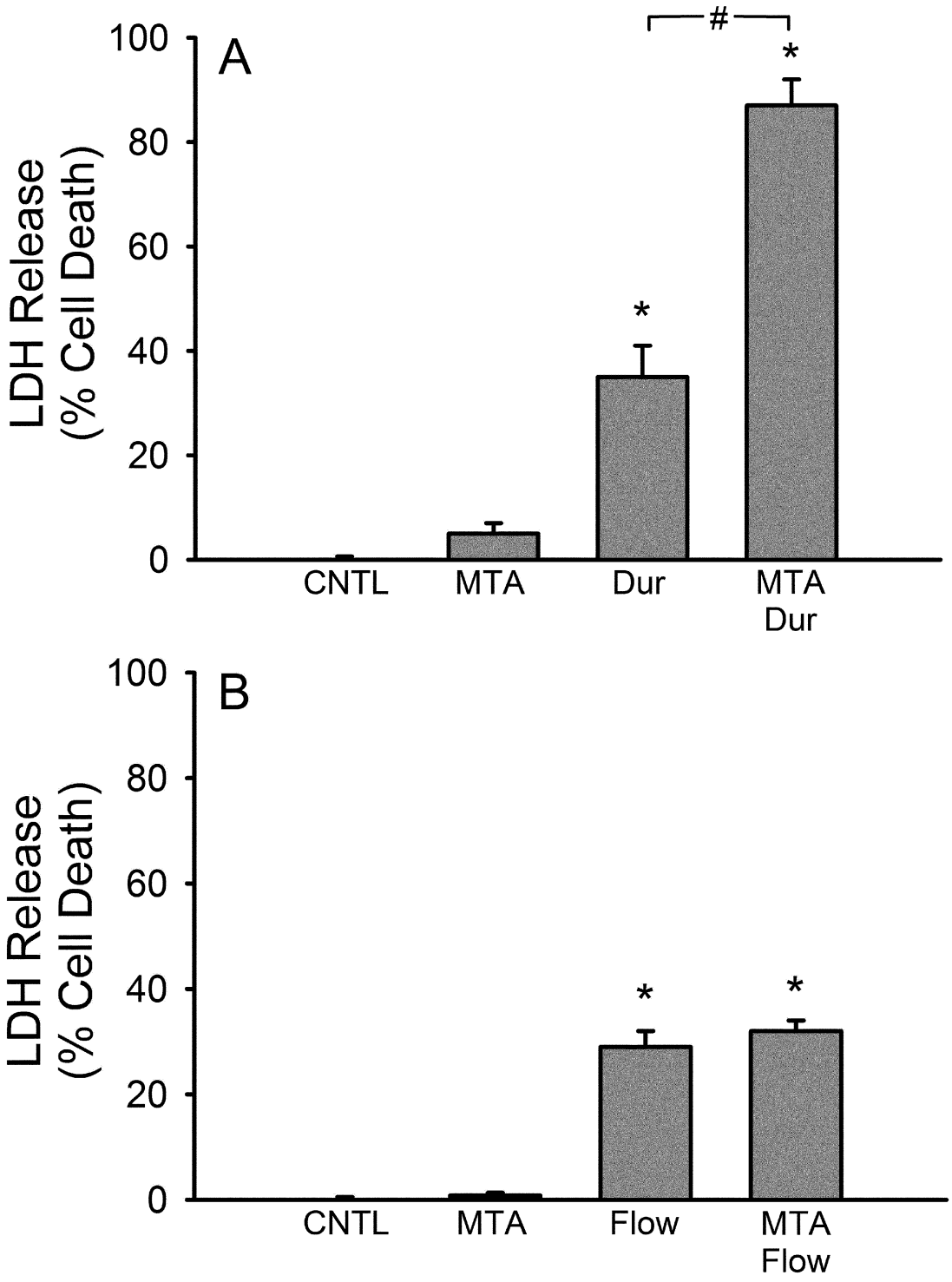

Figure 6. Effect of ProRoot MTA on toxicities of Durafill VS and Flow Line. ProRoot MTA significantly enhanced the toxicity of Durafill VS, while it had no effect on the toxicity of Flow Line ($p<0.05$). * denotes significant difference from control; # denotes significant difference between samples.

CHAPTER V

DISCUSSION

This in vitro study is the first to investigate the toxic effects of pulp capping materials and restorative composites when applied consecutively to the same dental pulp cells. Cultured pulp cells were exposed to either Dycal, a CH-based cement, or ProRoot MTA followed by either Flow Line or Durafill VS, two popular composite materials. The results indicate the ability of CH- and MTA-based cements to differentially alter the toxicities of these composites: Dycal exposure offered some protection from the toxicity of Flow Line, and ProRoot MTA exposure augmented the toxicity of Durafill VS.

We first tested the relative toxicities of Dycal and MTA using the LDH release assay. Dycal was toxic while MTA was not. Furthermore, Dycal's toxicity was dose-dependent, while MTA was nontoxic even at doses where Dycal was extremely toxic. Clinically, capping materials should be applied conservatively to the precise area of pulp exposure. Dentin covered with Dycal or MTA will not chemically bond to composite, leaving gaps at the tooth-restoration interface that weaken the integrity of the restoration and allow bacteria to penetrate. Based on our findings, it would seem another potential benefit of conservative Dycal application would be to limit its toxicity to the pulp.

Next, the LDH release assay was used to determine the toxicities of Flow Line and Durafill VS after pulp cell exposure to either Dycal or ProRoot MTA. Small, nontoxic pieces of Dycal and MTA were used to ensure cell death was primarily due to the addition of the composite. While MTA exposure enhanced the toxicity of Durafill VS, no effect was observed between MTA and Flow Line. Conversely, Dycal inhibited the toxicity of Flow Line but had no effect on the toxicity of Durafill VS. These findings

are counter to MTA's reputation for excellent biocompatibility and Dycal's inherent toxicity. If reproducible in the clinical setting, such interactions could contribute to overwhelming the pulp's defense mechanisms, especially when there is already inflammation present due to other causes. The pulp is able to recover from inflammation due to dental material toxicity, bacterial contamination, trauma, and iatrogenic damage, but the extent of total inflammation from all sources is what determines the pulp's ability to make a full recovery and form reparative dentin.

The results of this study call for explanation of the differences in composition between the composite materials used. Durafill VS is a microfilled flowable composite produced on the basis of Bis-GMA, TEGDMA, and UDMA. Flow Line is classified as a hybrid flowable composite containing Bis-GMA and TEGDMA monomers. When these monomers were studied on mouse fibroblasts in vitro, the toxicity rank order was as follows: Bis-GMA (most toxic) > UDMA > TEGDMA (least toxic) (19). When combinations of these monomers were tested, synergistic, additive, and antagonistic interactions were found, depending on the constituents and their concentrations (19). It is therefore reasonable to propose the potential for MTA and CH to undergo similar types of interactions with composite monomers, which may help explain the findings of the current study.

As a novel investigation, the intent of this study was to determine if the cytotoxic response of pulp cells exposed to capping materials and composites was additive, synergistic or antagonistic. Tests performed on cell cultures lack the complex and dynamic nature of in vivo experiments and cannot be translated directly into clinical practice. For example, the protective and defensive features of the pulpal inflammatory

response were largely unaccounted for in this study, as was the barrier effect of the pulp cap to pulpal contact with the restorative material. Clinically, the pulp cap is placed in a layer between the pulp and restoration. The toxicities of the restorative materials tested in this study, wherein composites were placed in direct contact with pulp cells, may therefore be more robust than those attained clinically.

The results of this study are most relevant if compounds leached from composites are able to breach the pulp space by navigating through or around a pulp cap. While no studies have investigated microleakage of capping materials to methacrylate monomers leached from composites or the amount of monomer necessary to elicit a cytotoxic response, many studies have identified deficiencies in the physical properties of pulp capping materials that may allow leakage to occur. Calcium silicate based cements such as MTA demonstrate a high degree of porosity and solubility, which increase significantly when higher water-to-powder ratios are used to mix the cement (51, 63, 64). Porosity and solubility are significantly less problematic in CH-based cements like Dycal (51). An in vitro study comparing the porosities and solubilities of Dycal and ProRoot MTA at 70% of final setting time found Dycal to be significantly less porous (9% vs. 29% for ProRoot MTA) and less soluble (5% vs. 11% for ProRoot MTA) (65). Another disadvantage of MTA in this regard is its prolonged initial setting time of up to 4 hours with a maturation period that persists for days, weeks, or longer (10). Freshly placed MTA is highly susceptible to dislodgement and dissolution, and the seal it provides with adjacent tooth structure is weak (66). For this reason, a hard-setting liner is frequently placed over unset MTA to provide adequate pulpal seal and protect from bacterial microleakage while the tooth is being restored. Interestingly, many liners used for this

purpose are resin-based and capable of leaching toxic methacrylate monomers (50). Once MTA has achieved initial setting, its protective advantage against these events seems to improve greatly. An in vitro study that compared fluid leakage values of completely set Dycal and ProRoot MTA placed on perforated dentin discs found significantly less fluid conductance through ProRoot MTA (67). The excellent seal formed between set MTA and surrounding tooth structure is likely to account for this difference, as CH-based cements lack inherent adhesive properties that may contribute to significant leakage.

Outside of deficiencies within the pulp cap itself, methacrylate monomers are also capable of reaching the pulp by diffusing through dentin tubules (68, 69). Adhesive resins applied to dentin thicknesses less than 0.5 mm are capable of causing chronic pulpal inflammation, confirming the ability of these leached compounds to diffuse through dentin in quantities great enough to elicit a toxic response (70). Therefore, the thickness of the dentin lateral to a pulp exposure is an important consideration when choosing a restorative material and limiting its toxicity to the pulp. The results of the current study indicate that the choice of pulp capping material may be another variable worthy of consideration when limiting the toxicity of the restorative material is an important goal of treatment.

The LDH release assay was used in this study to assess cell death, and by extension, material toxicity. LDH is normally found intracellularly and is released into extracellular spaces only when the cell membrane is no longer intact, indicating cell death. While LDH release is a reliable marker of cell death, cell death is an incomplete measure of tissue viability. When cytotoxicity is used to assess overall tissue health and vitality, all modes of potential cellular and tissue impairment should be considered. The

effects of materials on cell metabolism, as measured by the MTT assay, for example, would be useful for evaluating the functionality and hence the viability of the surviving cells. Findings from more than one assay would not only allow for a more complete evaluation of cytotoxic effects but would also enhance the integrity of the results. It should also be emphasized that the results of these tests are insufficient for predicting clinical outcomes. Rather, the findings are meant to serve as a preliminary basis for further investigation.

The ability of ProRoot MTA and Dycal to alter the toxicities of composites used in this study highlights the need for further testing to identify the mechanisms involved and clinical implications of our findings. Detailed knowledge of the interactions between pulp capping and composite materials is important for outlining their appropriate use and developing new materials with superior properties. Suggestions for further research include testing the effects of MTA and CH-based cements on the toxicities of various monomers found in composite materials including Bis-GMA, TEGDMA, UDMA, HEMA, etc. Such a study would help determine whether our results could be further ascribed to certain monomeric constituents, and in doing so, allow for broader application of our findings to entire classes of composite materials vs. only those used in this study. Ultimately, investigation in vivo would enable simulation of the clinical scenario with more relevant outcome measures.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Dental pulp cells underwent viability screening via the LDH release assay to determine the effects of Dycal, a CH-based cement, and ProRoot MTA on the toxicities of two composite restorative materials, Flow Line and Durafill VS.

Dycal was toxic, while MTA was not. Dycal's toxicity was correlated with the amount of material placed in culture, while MTA was nontoxic even in amounts much larger than those at which Dycal was highly toxic.

Exposure of pulp cells to Dycal significantly reduced the toxicity of Durafill but had no effect on the toxicity of Flow Line. Exposure of pulp cells to MTA significantly increased the toxicity of Flow Line but had no effect on Durafill toxicity.

Conclusions:

1. While MTA is nontoxic by itself, here we have demonstrated its potential to selectively enhance the toxicity of some composite materials, which may hinder the pulp's ability to recover from the insult of exposure and any preexisting inflammation. This highlights the need for a deeper understanding of MTA's interactions with other frequently used dental materials.
2. While Dycal is inherently toxic, it may protect the pulp from the toxicities of certain composite materials. If reproducible in vivo, such an effect would elucidate an additional benefit to CH-based materials for direct pulp capping that has been unaccounted for in the debate over which pulp capping material (CH vs. MTA) possesses superior properties.

3. Further investigation is needed to identify the existence of these findings in vivo and the extent to which they are clinically relevant.

REFERENCES

1. Schröder U. Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. J Dent Res. 1985;64:541-8.

2. Dammaschke T, Leidinger J, Schäfer E. Long-term evaluation of direct pulp capping—treatment outcomes over an average period of 6.1 years. Clin Oral Investig. 2010;14(5):559-67.

3. Aguilar P, Linsuwanont P. Vital pulp therapy in vital permanent teeth with cariously exposed pulp: A systematic review. J Endod. 2011;37(5):581-7.

4. Matsuo T, Nakanishi T, Shimizu H, Ebisu S. A clinical study of direct pulp capping applied to carious-exposed pulps. J Endod. 1996;22(10):551-6.

5. Mente J, Hufnagel S, Leo M, Michel A, Gehrig H, Panagidis D, Saure D, Pfefferle T. Treatment outcome of mineral trioxide aggregate or calcium hydroxide direct pulp capping: Long-term results. J Endod. 2014;40(11):1746-51.

6. Modena KC, Casas-Apayco LC, Atta MT, Costa CA, Hebling J, Sipert CR, Navarro MF, Santos CF. Cytotoxicity and biocompatibility of direct and indirect pulp capping materials. J Appl Oral Sci. 2009 Nov-Dec;17(6):544-54.

7. Bergenholtz G. Evidence for bacterial causation of adverse pulpal responses in resin-based dental restorations. Critical Reviews in Oral Biology & Medicine. 2000;11(4):467-80.

8. Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J. 2011;44(8):697-730.

9. Schmalz G, Arenholt-Bindslev D. Biocompatibility of dental materials. . 2009

10. Roberts HW, Toth JM, Berzins DW, Charlton DG. Mineral trioxide aggregate material use in endodontic treatment: A review of the literature. Dental Materials. 2008;24(2):149-64.

11. Aeinehchi M, Eslami B, Ghanbariha M, Saffar A. Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: A preliminary report. Int Endod J. 2003;36(3):225-31.

12. Furey A, Hjelmhaug J, Lobner D. Toxicity of flow line, durafill VS, and dycal to dental pulp cells: Effects of growth factors. J Endod. 2010;36(7):1149-53.

13. Yasuda Y, Ogawa M, Arakawa T, Kadowaki T, Saito T. The effect of mineral trioxide aggregate on the mineralization ability of rat dental pulp cells: An in vitro study. J Endod. 2008;34(9):1057-60.

14. Poggio C, Ceci M, Beltrami R, Dagna A, Colombo M, Chiesa M. Biocompatibility of a new pulp capping cement. Ann Stomatol. 2014;5(2):69.

15. Poggio C, Arciola CR, Beltrami R, Monaco A, Dagna A, Lombardini M, Visai L. Cytocompatibility and antibacterial properties of capping materials. The Scientific World Journal. 2014;2014

16. Gandolfi M, Taddei P, Tinti A, Prati C. Apatite‐forming ability (bioactivity) of ProRoot MTA. Int Endod J. 2010;43(10):917-29.

17. Guven G, Cehreli ZC, Ural A, Serdar MA, Basak F. Effect of mineral trioxide aggregate cements on transforming growth factor β 1 and bone morphogenetic protein production by human fibroblasts in vitro. J Endod. 2007;33(4):447-50.

18. About I, Camps J, Mitsiadis TA, Bottero M, Butler W, Franquin J. Influence of resinous monomers on the differentiation in vitro of human pulp cells into odontoblasts. J Biomed Mater Res. 2002;63(4):418-23.

19. Ratanasathien S, Wataha J, Hanks C, Dennison J. Cytotoxic interactive effects of dentin bonding components on mouse fibroblasts. J Dent Res. 1995;74(9):1602-6.

20. Accorinte, Maria de Lourdes Rodrigues, Loguercio AD, Reis A, Muench A, de Araújo VC. Adverse effects of human pulps after direct pulp capping with the different components from a total-etch, three-step adhesive system. Dental Materials. 2005;21(7):599-607.

21. Pashley DH, Walton RE, Slavkin HC. Histology and physiology of the dental pulp. Ingle JI, Bakland LK.Endodontics. 2002;5:43-5.

22. Holland R, de Souza V, de Mello W, Nery MJ, Bernabé PF, Otoboni Filho JA. Permeability of the hard tissue bridge formed after pulpotomy with calcium hydroxide: A histologic study. J Am Dent Assoc. 1979;99(3):472-5.

23. Caplan DJ, Cai J, Yin G, White BA. Root canal filled versus Non‐Root canal filled teeth: A retrospective comparison of survival times. J Public Health Dent. 2005;65(2):90-6.

24. Lim KC, Kirk EEJ. Direct pulp capping: A review. Dental Traumatology. 1987;3(5):213-9.

25. Fouad A, Torabinejad M, Walton RE. Endodontics: Principles and practice. 2008

26. Chen E, Abbott PV. Dental pulp testing: A review. International journal of dentistry. 2009;2009

27. Gopikrishna V, Pradeep G, Venkateshbabu N. Assessment of pulp vitality: A review. International Journal of Paediatric Dentistry. 2009;19(1):3-15.

28. Seltzer S, Bender I, Ziontz M. The dynamics of pulp inflammation: Correlations between diagnostic data and actual histologic findings in the pulp. Oral Surgery, Oral Medicine, Oral Pathology. 1963;16(8):969-77.

29. Ghoddusi J, Forghani M, Parisay I. New approaches in vital pulp therapy in permanent teeth. Iran Endod J. 2014 Winter;9(1):15-22.

30. American Association of Endodontists. Guide to Clinical Endodontics. 5th ed. Chicago: ; 2013. 10 p.

31. Milosevic A. Calcium hydroxide in restorative dentistry. J Dent. 1991;19(1):3-13.

32. Lin P, Huang S, Chang H, Chi L. The effect of rubber dam usage on the survival rate of teeth receiving initial root canal treatment: A nationwide population-based study. J Endod. 2014;40(11):1733-7.

33. Sidhu S, Schmalz G. The biocompatibility of glass-ionomer cement materials. A status report for the american journal of dentistry. Am J Dent. 2001;14(6):387-96.

34. Hermann B. Calcium hydroxid als mittelzurn, behandeln und fullen von wurzelkanalen [thesis]. Germany: University of Würzburg. 1920

35. Stanley H, Pameijer C. Dentistry's friend: Calcium hydroxide. Oper Dent. 1997;22(1):1.

36. Cox C, Bergenholtz G, Heys D, Syed S, Fitzgerald M, Heys R. Pulp capping of dental pulp mechanically exposed to oral microflora: A 1–2 year observation of wound healing in the monkey. Journal of Oral Pathology & Medicine. 1985;14(2):156-68.

37. Hilton TJ. Keys to clinical success with pulp capping: A review of the literature. Oper Dent. 2009;34(5):615.

38. Siqueira J, Lopes H. Mechanisms of antimicrobial activity of calcium hydroxide: A critical review. Int Endod J. 1999;32(5):361-9.

39. Cox CF, Suzuki S. Re-evaluating pulp protection: Calcium hydroxide liners vs. cohesive hybridization. J Am Dent Assoc. 1994;125(7):823-31.

40. Torabinejad M, Hong C, McDonald F, Ford TP. Physical and chemical properties of a new root-end filling material. J Endod. 1995;21(7):349-53.

41. Torabinejad M, Parirokh M. Mineral trioxide aggregate: A comprehensive literature review—part II: Leakage and biocompatibility investigations. J Endod. 2010;36(2):190-202.

42. Pistorius A, Willershausen B, Marroquin BB. Effect of apical root‐end filling materials on gingival fibroblasts. Int Endod J. 2003;36(9):610-5.

43. Faraco IM, Holland R. Response of the pulp of dogs to capping with mineral trioxide aggregate or a calcium hydroxide cement. Dental Traumatology. 2001;17(4):163-6.

44. Nair P, Duncan H, Pitt Ford T, Luder H. Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with mineral trioxide aggregate: A randomized controlled trial. Int Endod J. 2008;41(2):128-50.

45. Briso AL, Rahal V, Mestrener SR, Dezan Junior E. Biological response of pulps submitted to different capping materials. Braz Oral Res. 2006 Jul-Sep;20(3):219-25.

46. Witherspoon DE. Vital pulp therapy with new materials: New directions and treatment perspectives—permanent teeth. J Endod. 2008;34(7):S25-8.

47. Parirokh M, Torabinejad M. Mineral trioxide aggregate: A comprehensive literature review—part I: Chemical, physical, and antibacterial properties. J Endod. 2010;36(1):16-27.

48. Cox C. Biocompatibility of dental materials in the absence of bacterial infection. Oper Dent. 1987;12(4):146.

49. Torabinejad M, Chivian N. Clinical applications of mineral trioxide aggregate. J Endod. 1999;25(3):197-205.

50. Dentsply TD. Directions for use: ProRoot™ MTA (mineral trioxide aggregate) root canal repair material. Literature from the manufacturer.Tulsa, OK: Dentsply Tulsa Dental. 1998

51. Gandolfi MG, Siboni F, Primus CM, Prati C. Ion release, porosity, solubility, and bioactivity of MTA plus tricalcium silicate. J Endod. 2014;40(10):1632-7.

52. Camilleri J, Pitt Ford T. Mineral trioxide aggregate: A review of the constituents and biological properties of the material. Int Endod J. 2006;39(10):747-54.

53. Miyashita H, Worthington HV, Qualtrough A, Plasschaert A. Pulp management for caries in adults: Maintaining pulp vitality. The Cochrane Library. 2007

54. Hilton T, Ferracane J, Mancl L. Comparison of CaOH with MTA for direct pulp capping A PBRN randomized clinical trial. J Dent Res. 2013:0022034513484336.

55. Zimmerli B, Strub M, Jeger F, Stadler O, Lussi A. Composite materials: Composition, properties and clinical applications. A literature review. Schweiz Monatsschr Zahnmed. 2010;120(11):972-86.

56. Huang F, Chang Y. Cytotoxicity of resin-based restorative materials on human pulp cell cultures. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2002;94(3):361-5.

57. Hanks C, Strawn S, Watahai J, Craig R. Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res. 1991;70(11):1450-5.

58. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences. 2000;97(25):13625-30.

59. Cabrera S, Barden D, Wolf M, Lobner D. Effects of growth factors on dental pulp cell sensitivity to amalgam toxicity. dental materials. 2007;23(10):1205-10.

60. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods. 1987;20(1):83-90.

61. Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: Validity for neuronal apoptosis? J Neurosci Methods. 2000;96(2):147-52.

62. Camargo S, Camargo C, Hiller K, Rode S, Schweikl H, Schmalz G. Cytotoxicity and genotoxicity of pulp capping materials in two cell lines. Int Endod J. 2009;42(3):227-37.

63. Fridland M, Rosado R. Mineral trioxide aggregate (MTA) solubility and porosity with different water-to-powder ratios. J Endod. 2003;29(12):814-7.

64. Fridland M, Rosado R. MTA solubility: A long term study. J Endod. 2005;31(5):376-9.

65. Gandolfi MG, Siboni F, Botero T, Bossu M, Riccitiello F, Prati C. Calcium silicate and calcium hydroxide materials for pulp capping: Biointeractivity, porosity, solubility and bioactivity of current formulations. J Appl Biomater Funct Mater. 2015 Jan-Mar;13(1):43-60.

66. Schmitt D, Lee J, Bogen G. Multifaceted use of ProRootTM MTA root canal repair material. Pediatr Dent. 2001;23(4):326-30.

67. Yalçin M, Barutcigil Ç, Sisman R, Yavuz T, Oruçoglu H. Evaluation of the sealing ability of pulp capping agents against leakage on direct pulp capping with a computerized fluid filtration meter. Journal of Restorative Dentistry. 2014;2(1):46.

68. Pashley D. Consideration of dentine permeability in cytotoxicity testing. Int Endod J. 1988;21(2):143-54.

69. Bouillaguet S, Wataha JC, Hanks CT, Ciucchi B, Holz J. In vitro cytotoxicity and dentin permeability of HEMA. J Endod. 1996;22(5):244-8.

70. Hebling J, Giro E, Costa C. Human pulp response after an adhesive system application in deep cavities. J Dent. 1999;27(8):557-64.