pH Change Following Smear Layer Removal and Final Rinse with 95% Ethyl Alcohol Using Calcium Hydroxide Medicament or BC Sealer

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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October 2016

Acknowledgements

Dr. McClanahan, Dr. Roach, Dr. Barsness, and Dr. Bowles:
Thank you for your dedication to this program and to my educational experience. Your mentorship, guidance, and support have been invaluable and mean the world to me.

Dr. Aparicio and Scott Lunos: Thank you for your assistance in producing this masters thesis

To Dr. Rhett Finley, Philip McKenzie, and Amber Zedler: Thank you for your friendship, encouragement, and understanding through many ups and downs. You have all helped me in ways known and unknown to reach a much anticipated finish line.

Dedication

This thesis is dedicated to my loving wife Rachael and daughter Catherine. Your endless sacrifice made it possible for me to accomplish the goals I set, in the face of much unanticipated adversity. I love you very much and promise that my time and energy going forward will be focused on our family.

Thank you to my family for your understanding and support through yet another educational adventure. Your love, guidance, and mentorship have been so appreciated through the years. Although I will be a lifelong student, my formal education has finally come to an end.

ABSTRACT

Introduction: An *in vitro* investigation of pH change at the external root surface after final rinse of 95% ethyl alcohol using Ultracal XS, Calasept Plus, or obturation with BC sealer.

Methods: 80 single rooted extracted human teeth were decoronated with a standardized root length of 15mm. All root were instrumented to size F4 using the ProTaper Gold file system. Defects were made on the facial and lingual root surfaces to remove cementum. Teeth were then randomized into treatment groups based on medicament used and final rinse with or without ethanol. Negative controls received no medicament. Full wax control groups received medicaments but were completely sealed with wax. After final rinses roots were filled with Ultracal XS, Calasept Plus, or BC sealer. Negative controls received no medicaments. Roots were sealed at coronal and apical ends using two layers of sticky wax and were placed in a 4mL lab vial containing 2mL of sterile saline. pH was measured days 0,1,2,4,7,9,12,16,22,28. Tooth roots were sectioned and remaining dentin thickness (RDT) was measured to correlate RDT and pH values. A multiple linear regression model was used to estimate the effects of medicament, ethanol, interaction of medicament and ethanol, as well as a continuous variable of RDT. Pairwise comparisons were completed to establish differences within groups.

Results: It appears that ethanol may have an effect on the rate of pH change in the Calasept Plus group. Trends towards a faster rise in pH were noted in both ethanol positive calcium hydroxide groups. BC sealer maintained a pH higher than negative controls for the first 12 days before dropping to baseline levels. RDT was found to affect pH levels, with greater thicknesses correlating to lower pH values. Maximum pH values were reached between days 12-16 after which a decline was noted in each group, indicating a replenishing of medicament may be necessary to maintain pH levels at the external root surface.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Calcium hydroxide (CH) is the recommended intracanal medicament for treatment of external inflammatory resorption (Hammarstrom, et al. 1986). Multiple applications exist for calcium hydroxide in endodontics. This medicament can be used to induce hard tissue formation, dissolve organic tissue, kill microorganisms, and to help arrest the resorptive process (Ghose, et al. 1987; Hasselgren, et al. 1988; Bystrom, et al.1985; Trope 2002). Removal of necrotic tissue from the pulp canal space appears to be of primary importance in managing inflammatory resorption (Dumsha and Hoveland 1995). Long-term CH has been advocated in cases where resorption has persisted for greater than two weeks before initiation of pulpal debridement (Trope, et al. 1995).

Resorption is a common sequelae of dental trauma (Trope, et al. 1995). Damage to the root surface will trigger initiation of surface resorption. If areas of surface resorption communicate with infected pulp tissue, external inflammatory resorption often ensues (Andreasen 1985). Resorption leads to loss of root structure, which may severely compromise prognosis for retention of the traumatized tooth or teeth (Tronstad 1988). Goals of treatment in these cases are to remove the infected tissue from the root canal, which acts as a primary driver of resorption, and to disrupt osteoclastic activity at the external root surface (Trope 2002).

Increased pH caused by hydroxyl ion diffusion through root dentin is thought to arrest the resorptive process (Tronstad, et al. 1981). There are several barriers that may inhibit successful diffusion of hydroxyl ions from CH medicaments (Fuss, et al., 1989; Calt, et al., 1999). Presence of smear layer dramatically reduces diffusion through dentin (Pashley, et al., 1988). Wang and Hume have confirmed that dentin has a significant buffering capacity (Wang and Hume 1988). When smear layer is removed, several investigations have demonstrated successful diffusion of hydroxyl ions to the external root surface (Foster, et al., 1993; Nerwich, et al.,1993; Crumpton, et al., 2005). The types and volumes of irrigants used have an effect on the degree of ion diffusion (Saif, et al., 2008). Because of the barriers to ion diffusion listed above, the rise in pH at the external root surface is delayed and can take up to three weeks to reach maximum levels (Nerwich, et al., 1992).

CH is the medicament of choice when treating EIR (Anderrson, et al., 2012). There are, however, several concerns with use of a CH as an intracanal medicament after traumatic injures. Hammarstrom and Lengheden both reported ankylosis as a result of intracanal CH placement after avulsions in animal models (Hammarstrom, et al., 1986; Lengheden, et al., 1990). Long-term calcium hydroxide has also been linked to weakening of root dentin and increased risk for root fracture (Andreasen, et al. 2002; Cvek 1992). Patient non-compliance and poor follow-up may also be considered a risk factor of long term CH therapy (Trope, et al. 1995; Andreasen 2002).

Endo Sequence BC Sealer (Brasseler USA, Savanah, GA) is a premixed injectable bioceramic paste indicated for use as a root canal sealer. This product is a calcium silicate based sealer that sets in the presence of moisture (Hess, et al., 2011). The manufacturer reports favorable handling properties and good tissue compatibility. The sealer is alkaline, with reported pH of 12.0, which provides favorable antimicrobial properties as well as good tissue healing properties. Previous investigations have observed the ability of MTA to induce an alkaline pH change at the external root surfaces *in vitro* (Heward and Sedgley 2011, Hansen, et al. 2011). Only one investigation has reported on BC sealer's ability to affect pH at the external root surface (Dudeja, et al., 2015).

Ethanol has been added to endodontic irrigants to decrease surface tension and increase dentin wettability, which improves their contact with dentin and increases tubule penetration (Cunningham, et al., 1988). Stevens suggested a possible increase in dentin wettability when alcohol is used as a final rinse. He noted increased sealer penetrated after final rinse of 95% ethyl alcohol versus a NaOCl *in vitro* (Stevens, et al. 2006). No studies have evaluated the effect a final rinse of 95% ethyl alcohol may have on diffusion of CH medicaments. Increased dentin wettability after application of 95% ethyl alcohol may facilitate better contact of CH medicaments with root dentin. Increased contact and tubule penetration may allow a more immediate or dramatic clinical effect of the CH medicament.

When treating external inflammatory resorption it is important to consider the dynamics of complete removal of infected pulp tissue, and resolution of the resorptive process. An expedited time frame may decrease risk for fracture related sequelae and help to improve patient compliance in treatment. Increasing the rate and magnitude of pH change at the external root surface may allow for more rapid and predictable treatment of external inflammatory resorption experienced after dental trauma. Likewise, immediate obturation with a bioceramic sealer may fulfill many or all of the clinical and biologic objectives in treating external root resorption in a single visit treatment model.

The purpose of this study is to determine the effect of a final rinse of 1mL 95% ethyl alcohol on the rate and magnitude of pH change at the external root surface after smear layer removal when using premixed calcium hydroxide medicaments UltraCal XS®, Calasept Plus® or single visit treatment model using EndoSequence BC sealer.

LITERATURE REVIEW

Calcium Hydroxide

CH is a white odorless powder with a molecular weight of 74.08 and pH of 12.5-12.8. The strong base has a low solubility (1.2gL-1 at 25°C) that decreases with a rise in temperature. The dissociation coefficient of CH is 0.17, meaning it will slowly dissociate into Ca^{2+} and OH^- ions over time in an aqueous environment. This property makes CH an ideal medicament for long-term applications (Rehman, et al. 1996).

Herman introduced CH as a direct pulp-capping agent in the 1920's (Herman 1920). It has since been widely used as an intracanal medicament based on a variety of applications. CH is used to promote hard tissue formation (Ghose, et al. 1987; Yates 1988; Binnie and Rowe 1983), it demonstrates effective antimicrobial action (Bystrom, et al. 1985; Safavi and Nichols 1993), it aids in the dissolution of organic tissue (Hasselgren, et al., 1988), and has been used as an effective means to control inflammatory root resorption (Tronstad, et al., 1981; Trope 2002).

Biologic properties of CH are due to the dissociation into Ca^{2+} and OH^- ions in an aqueous environment. The hydroxyl ions are highly oxidant free radicals that react with

surrounding tissue. These tissue interactions confer antimicrobial properties of CH when used as a canal dressing. Hydroxyl ions affect bacterial cells by disrupting the cytoplasmic membrane, denaturing proteins, and damaging DNA (Siqueira and Lopes 1999). These actions account for the lethal effects of CH on bacterial cells in the root canal (Bystrom, et al., 1985; Stuart, et al., 1991).

CH can also reduce the quantity of lipopolysaccharide (LPS) by hydrolysis of an ester bond in the fatty acid chains of the lipid A moiety (Buck, et al. 2001). In a dog model, Silva demonstrated that treatment of infected canals with CH detoxified endotoxin, which limited apical inflammation (Silva, et al. 2002). It is also thought that this may be a mechanism by which CH helps prevent osteoclastogenesis. Jiang found that reduction of LPS by CH significantly reduced formation of osteoclasts stimulated by endotoxin *in vitro* (Jiang, et al. 2003).

Diffusion of hydroxyl ions to the external root surface increases pH in areas of resorption. Tronstad demonstrated pH change in root dentin of teeth treated with CH (Tronstad, et al. 1981). The alkaline environment is not suitable for bacteria or dentin resorbing cells (Hammarstrom, et al. 1986). The change of pH in resorptive lacunae may necrotize cells associated with resorption, neutralize acid formation from osteoclasts and macrophages, as well as disrupt activity of collagenases and hydrolase activity. CH effectively prevents continuation of external resorption and may influence a repair response by activating enzymes linked to mineralization (Tronstad 1988).

The vehicle used to carry CH into the canal can influence the physical and antimicrobial properties of the medicament (Fava and Saunders 1999). According to Fava the ideal vehicle would: 1. Allow a gradual and slow ionic release: 2. Allow slow diffusion in the tissues with low solubility in tissue fluids: 3. Have no adverse effect on the induction of hard tissue deposition. Three classes of vehicles that have been described in the literature: 1. Oily 2. Viscous 3. Aqueous (Fava and Saunders 1999). Safavi measured dissociation of CH in two nonaqueous vehicles and concluded that use of either glycerin or propylene glycol as a mixing vehicle may decrease effectiveness of the CH dressing due to a decrease or delay in hydroxyl ion diffusion (Safavi, et al. 2000). Aqueous vehicles such as saline, local anesthetic, or methylcellulose have a superior

ability to allow dissociation of CH (Blancet, et al. 1998). Thinner pastes of CH demonstrated more favorable reduction in *E. faecalis* from dentinal tubules. Therefore, viscosity of the paste may also influence antimicrobial actions of the medicament (Behnen, et al. 2001). A disadvantage of an aqueous vehicle is that it can be rapidly solubilized. Dressings may need to be replaced more often when employing this type of CH vehicle in certain clinical scenarios (Fava and Saunders 1999).

In this experiment, two commonly used aqueous premixed CH dressings were compared. UltraCal XS (Ultradent South Jordan, UT) and Calasept Plus (Dentsply Sirona, New York, NY) are both available in premixed syringes. The manufacture of UltraCal XS reports a CH concentration of 35 Wt % with Barium Sulfate added as a radiopacifier at a concentration <20 Wt. %. The vehicle is described as aqueous, but is not specified by the manufacturer. Past studies with previous versions of the product report the use of aqueous methylcellulose as a delivery vehicle (Blancet, et al., 1998). The Material Safety Data Sheet lists the pH of UltraCal XS between 12.0 and 13.0.

Calasept Plus (Dentsply, Sirona, New York, NY) manufacturer reports a CH concentration of 41-46% and a Barium Sulphate concentration of 5-10%. The mixing vehicle is listed as Ringer solution >48%, and the pH is reported to be 12.4. Both of these materials are convenient to apply, and have been previously demonstrated to affect pH change and have adequate antimicrobial properties (Heward and Sedgley 2011; Chamberlain, et al., 2009; Silva, et al., 2002; Zmener et al., 2007). The common use and favorable properties of these specific dressings makes their inclusion in this study clinically applicable.

Bioceramic Sealer

Bioceramic materials have been recently introduced in endodontics as root canal sealers (Zhang W., et al. 2009). Endosequence BC sealer (Brasseler USA, Savannah GA) is a calcium silicate premixed sealer that is reported to contain zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents (Hess, et al. 2011). This sealer is hydrophilic and is designed to use moisture from the dentinal tubules to initiate the setting reaction. The manufacturer reports a setting time of

4 hours, but this can be extended up to 168 hours depending on moisture content of the setting environment (Loushine, et al. 2011). Upon setting, the sealer has an ability to form a hydroxyapatite precipitate, which promotes a favorable environment for remineralization of diseased or damaged surfaces (Loushine, et al. 2011). The interaction of tricalcium silicate-based sealer with tissue fluids demonstrates an exchange of calcium and silicon from material to dentin, establishing a basis for bioactivity of the sealer (Xuereb, et al., 2015). Available literature supports the biocompatibility of the BC sealer in cell viability models (Loushine, et al. 2011; Zhou, et al. 2015). The pH is reported in the literature at maximum levels of 11.21 after 10 days *in vitro* (Candiero, et al. 2012). This alkaline pH is thought to confer favorable antimicrobial effects that may be extended when in contact with dentin (Zhang H., et al. 2009; Wang, et al. 2014). There is some evidence that use of BC sealer may increase fracture resistance of roots compared to other commonly used sealers (Ghoneim, et al., 2011; Dudeja, et al., 2015). The ease of application and favorable biological and physical properties make this an attractive addition to the sealers available for endodontic use. At this time, one study has been published evaluating pH change at the external root surface after application of BC sealer in a dentin model (Dudeja, et al., 2015). This model however, did not include obturation with gutta-percha as advocated by the manufacturer.

Resorption:

Resorption is a condition associated with either a physiologic or a pathologic process resulting in a loss of dentin, cementum and/or bone (AAE Glossary of Terms). Typically the mineralized tissues of the permanent dentition are not resorbed. Predentin and odontoblasts protect tissue adjacent to the canal, while precementum and cementoblasts protect the external root surface against physiologic resorption that regularly takes place in surrounding alveolar bone (Hammarstrom and Lindskog1985; Wedenberg and Lindskog 1987). When resorption does occur, loss of tooth structure is irreversible with the exception of transient surface resorption (Bakland 1992). Physiologic resorption in teeth is limited to the exfoliation of the primary dentition. Pathologic resorption occurs when protective external surfaces have been disrupted or

damaged, allowing colonization by multinucleated resorptive cells (Lindskog, et al., 1987). The resorptive process has been linked to several conditions including periapical inflammation, trauma, tumors and cysts, excessive mechanical or occlusal forces, and impacted teeth. Understanding the underlying mechanism and etiology of the disease process is imperative to successful diagnosis and subsequent treatment interventions.

Osteoclasts are multinucleated bone resorbing cells that are derived from hematopoietic cells of the monocyte-macrophage lineage (Pierce et al., 1989). The cells are characterized by several unique features. These multinucleated clastic cells have specialized membrane structures, ruffled boarders, and clear zone that work to facilitate the resorptive process (Pierce, et al., 1991). Dentinoclasts are very similar if not the same as their osteoclast counterparts. The two cells can be distinguished based on slight morphological differences, as dentinoclasts tend to contain fewer nuclei, and have smaller or no clear zones. Both stain positive for tartrate resistant acid phosphatase (TRAP) a well-described marker for osteoclasts (Ne et al., 1999).

Odontoclasts are regulated by a complex network of signals that control the activation and suppression of the resorptive process. Systemic factors related to their activation are primarily parathyroid hormone (PTH) and vitamin D (1,25 dihydroxycholecalciferol). Calcitonin is a balancing control responsible for inhibiting resorption through release of osteoprotegrin (OPG), which competitively inhibits the activation of clastic cells (Ne, et al., 1999). Local factors including the inflammatory mediators IL-1, TNFα, and PGE, play rolls in the formation, activation, and regulation of osteoclasts (Kawashima and Stashenko 1999; Kawaguchi, et al., 1995). Although these factors do upregulate resorption, the activation of osteoclasts is controlled by an indirect mechanism involving RANK. Receptor Activator of Nuclear Factor K B (RANK), also known as TRANCE, is a surface receptor expressed by osteoclasts. This receptor is activated by RANK- ligand, which is released by osteoblasts when triggered by many of the previously listed local or system factors. RANKL is then primarily responsible for activating clastic cells (Tyrovola, et al., 2008).

Once activated osteoclasts and dentinoclasts can bind to mineralized tissues. After binding they form a sealed environment with a ruffled boarder. Proton pumps

mediate an acidic environment that dissolves inorganic mineral content. Vacuoles containing capthesin B, and acid phosphatase among other enzymes are released to facilitate breakdown of the organic collagen matrix (Pierce, et al., 1991). Typically the dental hard tissues are not involved in resorption due to a protective mechanism. There is evidence that cementum and possibly dentin act as an inhibitor of resorption by discouraging the binding of dentinoclasts to cementum (Wedenberg and Lindskog 1987).

The pathophysiology and clinical presentation of the different resorptive processes have been well described and reviewed in the literature (Bakland 1992, Trope 1992; Ne, et al., 1999; Andreasen 1981). There is, however, some variation in descriptive nomenclature of the different entities. In general terms, resorption of dental hard tissues can be classified based on the site, nature, and pattern of the process (Benenati 1997). The location of resorption is usually described as either internal (relating to the root canal wall) or external (relating to the external root surface) in nature. Terms including cervical and apical resorption help to further specify location of the process. Resorption can be described as either transient meaning limited or progressive meaning continuous (Tronstad 1988). Surface, inflammatory, and replacement resorption have been described as three separate, but common resorptive entities (Bakland 1992). Additional categories are described in the literature include invasive or cervical resorption, pressure resorption, and idiopathic resorption.

Mechanical injury to the protective outer root surface is required for initiation of external resorption (Andreasen 1985). Partial or complete loss of cementum and damage to the attachment apparatus facilitates local inflammation and access to mineralized root surfaces. These exposed root surfaces are colonized by multinucleated clastic type cells or dentinoclasts (Pierce 1989). The inflammatory response from initial injury to the tooth root facilitates initiation of surface resorption. This initial response tends to be self-limiting and will spontaneously resolve depending on the degree of root surface damage (Ne, et al., 1999). Repair with cementum-like tissue typically occurs within two to three weeks (Lindskog et al., 1983). This transient process is labeled surface resorption, and is most often insignificant from a clinical perspective. The resorption is not detectable radiographically, symptoms are not present and normal root contours are generally

maintained or reestablished through cemental repair. For these reasons no treatment interventions are required.

The transient process of surface resorption can give way to a progressive process if an additional stimulus is applied. This progressive type of resorption is called External Inflammatory Resorption (EIR), which is the focus of this project. Bacterial infection of the pulpal tissues is the primary stimulus that drives external inflammatory resorption (Andreasen 1981). Damage or loss of the protective cementum and attachment apparatus during injury facilitates communication between the root canal system and periodontal tissues through patent dentinal tubules (Andreasen 1985). Bacteria and their byproducts supply a continuous inflammatory drive that promotes continuous resorption (Pierce 1989). If the process is not controlled, roots can be completely resorbed in weeks or months (Andreasen, et al., 2007). EIR can occur anywhere along the tooth root, but it will most often occur at corners of root surfaces where attachment is damaged during traumatic dental injuries (Andreasen 1981). If the resorption occurs as a consequence of long-standing infected root canal systems alone, usually it occurs apically (Abbott 2016; Laux, et al. 2000).

EIR is associated with more severe traumatic dental injures. Avulsions, intrusions, and lateral luxations are more commonly associated as they are more likely to affect pulp vitality, as well as causing damage to the PDL (Andreasen, et al., 2006). After damage to the PDL, infected dental tissue provides the stimulus for EIR to progress. The incidence of external inflammatory resorption after dental trauma ranges from 5 to 18% (Crona-Larsson, et al., 1991) in luxation injuries and affects up to 30% of avulsed teeth that are replanted (Andreasen et al., 1995b). Apical inflammatory resorption affects up to 80% of teeth on a histologic level (Laux, et al., 2000). On radiographic exam, EIR is characterized by concave and sometimes ragged bowl shaped radiolucencies along the root surface with corresponding areas of resorbed alveolar bone (Ne, et al., 1999). The relative size of the lesions dictates our ability to detect the process, especially in two-dimensional radiography. Use of cone beam computed tomography allows a more sensitive localization process and increases the information we receive in terms of extent and location of the resorptive lesion (Bernardes, et al.,

2012). Initial radiographic signs of can be seen as early as 3 to 4 weeks after injury and are always seen within a year. The process tends to progress more quickly in younger age groups, likely due to the degree of patency of dentinal tubules (Andreasen 1981).

Internal resorption is thought to be less common than external resorption (Bakland 1992). It can also proceed in both transient or progressive type presentations, and has been linked strongly to traumatic luxation injuries. Necrotic tissue and vital tissue are both necessary to promote this type of resorption. Damage to the internal canal walls facilitates a similar colonization by clastic cells whose source is likely vital pulpal tissue. Bacterial contamination of necrotic tissue provides the prolonged stimulus to promote continuation of the resorptive process. Treatment of internal resorption is aimed at removal of any remaining tissue in the canal system, which will successfully negate the process (Ne, et al., 1999).

Replacement resorption and ankylosis are often referred to as the same condition in the literature (Bakland 1992) and are defined by the AAE glossary of terms as synonymous (AAE glossary of terms 2015). Ankylosis occurs after extensive necrosis of the periodontal ligament following dental trauma. There is a union of alveolar bone and external root surface with no intervening connective tissue (Ne, et al., 1999). It is a serious condition because involved teeth become incorporated into the alveolar bone remodeling process. Osteoclasts no longer distinguish between bone, cementum or dentin and teeth are therefore progressively resorbed (Bakland 1992). Replacement resorption is viewed by Tronstad as a “mistake”, and endodontic treatment interventions are unsuccessful in disrupting the process.

Clinically, ankylosis is identified by a lack of physiologic mobility and a special metallic sound on percussion. Radiographically the PDL space becomes absent, and ingrowth of alveolar bone will create a moth-eaten appearance (Tronstad 1988). Infraocclusion may be noted after some time, especially in a younger developing patient population (Malmgren and Malmgren 2002). Similar to EIR, replacement resorption or ankylosis can be transient or progressive in nature after traumatic injuries. Transient cases typically affect less than 20% of the root surface, and there seems to be a potential for reversal and reestablishment of a periodontal ligament between tooth and bone

(Tronstad 1988). When ankylosis is progressive, tooth structure is gradually resorbed and replaced by bone until no root structure remains. The severity of injury and age of the patient tend to affect rate of replacement resorption (Lindskog et al., 1985). More severe injuries and younger patients demonstrate a much faster rate of replacement resorption, whereas older patients may maintain affected teeth for longer periods of time.

External cervical resorption (ECR) originates at the external root surface. It is thought to initiate just apical to the epithelial attachment in the cervical region. Several terms have been used to describe the condition: invasive cervical resorption, supraosseous extracanal invasive resorption, peripheral inflammatory root resorption, etc. It is generally accepted that a similar breech in the protective non-mineralized layers of the external root surface must exist to allow binding of clastic cells to underlying dentin. Orthodontics, traumatic dental injuries, oral surgical procedures, and internal bleaching have been linked to this pathologic process as potential causes (Heithersay 1999). The progression or maintenance of the disease process has been a point of conflict in the literature. Heithersay promotes a benign proliferative fibrovascular or fibro-osseous disorder, while others view ECR as a disease process mediated by stimulation of microorganisms similar to EIR (Lin, et al., 2013).

Treatment Modalities:

Appropriate treatment for resorption varies by the type of resorption. Of the classifications reviewed previously, root canal therapy is only useful in treating inflammatory type resorption. Depending on the presentation of invasive cervical resorption, root canal therapy may be an appropriate treatment intervention (Bakland 1992). In most cases of replacement resorption, pressure resorption, or idiopathic resorption root canal therapy will not address the etiology of the resorption and therefore will not arrest the resorptive process (Bakland 1992; Ne, et al. 1999; Trope 2006). This experimental model is designed to simulate dentinal defects that may be present at the external root surface in EIR secondary to trauma. Discussion of treatment will focus on this specific disease entity and presentation.

Andreasen presents four conditions that must be met for inflammatory resorption to develop. An injury to the periodontal ligament must take place. In this case, the PDL and parts of the external root surface are physically disrupted by the injury and subsequent inflammation. During the initial inflammatory response located in the PDL surface resorption is initiated by clastic cells. Surface resorption must expose underlying dentinal tubules, which communicate with bacteria and necrotic tissue in the root canal system (Andreasen 1985). Bacteria from the root canal space and their byproducts are the driving force behind the initiation and progression of external inflammatory resorption (Andreasen 1981). Our treatments, therefore, are aimed at two targets. Elimination of bacteria from the root canal system is the primary objective. Treatment will also seek to arrest the inflammation and resorption present at the external root surface (Tronstad 1988).

It is possible to prevent external inflammatory resorption with appropriate treatment after traumatic injury (Abbott 2016). Removing the diseased pulp and placing a short term CH dressing is recommended by current IADT guidelines to help prevent EIR after avulsion injuries or severe luxation injuries in fully developed teeth (Andersson, et al., 2012). Typically, debridement is delayed 7-10 days and CH is used up to 1 month. This delay in treatment helps to avoid ankylosis, which is a possible side effect of CH application immediately after traumatic injury (Hammarstrom, et al., 1986). Additional intracanal medicaments have been studied including steroid and antibiotic dressings, and offer promising results in prevention of resorption while decreasing the risk for ankylosis when applied immediately after trauma (Pierce 1985; Bryson, et al 2002).

If the necrotic pulp tissue is removed in a timely fashion, there is evidence that predictable healing will occur without placement of an intracanal medicament. Dumsha and Cvek contend that elimination of the bacterial stimulus alone is adequate. They were able to demonstrate NSD in healing patterns between those groups immediately obturated with gutta-percha those that received CH medicament before obturation in animal avulsion models (Dumsha and Hoveland 1995; Cvek 1992). Many personal circumstances can impede timely management of traumatic dental injuries. Initiation of

EIR before treatment is more likely in cases of delayed evaluation. In teeth with established EIR, it may be more difficult to arrest the resorptive process and encourage external repair (Trope, et al., 1995). In these cases, the use of CH as an intracanal medicament plays a larger role in arresting resorption and encouraging repair (Tronstad, et al., 1981).

Tronstad demonstrated pH change in a monkey model at the external root surface when using CH as an intracanal medicament (Tronstad, et al. 1981). His assessment was that pH change occurred secondary to hydroxyl ion diffusion through the dentinal tubules. As previously discussed, CH demonstrates good antimicrobial properties (Bystrom, et al, 1985) and is capable of creating an environment that is unfavorable for clastic cells (Tronstad, et al., 1981). CH is also known to demonstrate a favorable response in hard tissue healing. The high pH environment may upregulate key enzymes that play a role in healing and mineralization (Hammarstrom, et al., 1986).

Maximum duration of treatment recommended by the International Association of Dental Traumatology in avulsion cases is 4 weeks after pulpal debridement (Andersson, et al. 2012). When EIR has been established for greater than two weeks there have been several recommendations for longer term CH dressing in order to facilitate arrest of resorption and encourage repair at the root surface (Tronstad 1988; Trope, et al., 1995). One possible reason for extended application of CH dressings is that CH does not immediately diffuse through root dentin, and therefore does not have its intended therapeutic effect immediately. Wang demonstrated that dentin has a great buffering capacity, which may initially limit diffusion of hydroxyl and calcium ions through dentinal tubules (Wang and Hume 1988). This concept was confirmed by Nerwich who found that it took minimally three weeks to reach maximum pH values at the external root surface in an extracted tooth model (Nerwich, et al 1993). Longer term dressings have been proposed to overcome this difficulty in achieving adequate pH change for desirable physiologic effects.

Long-term CH dressings may have unintended negative consequences. Patient compliance may decrease when asked to extend treatment intervals over several months rather than days or weeks (Andreasen, et al 2002). A more pressing concern is that CH

medicaments, when used for extended durations, may contribute to a weakening of the root dentin (Andreasen, et al., 2002; Cvek 1992, Sahebi, et al., 2010). Especially in teeth that are not fully developed this may put a patient at risk for tooth or root fracture. Cvek reported that 60% of immature permanent incisors experienced cervical root fracture on secondary trauma after long term CH therapy during initial treatment. In a recent study, Hawkins refutes the claim that longer-term dressings weaken radicular dentin (Hawkins, et al., 2015), but the majority of studies support dentinal weakening with applications of CH that extend over 30 day.

Because of these risk factors, it is prudent to investigate a methodology that may more efficiently and effectively promote necessary changes at the external root surface when using CH as an intracanal medicament. If dressings can be used in cases of established EIR for durations less than 1 month, then patient compliance may improve while also decreasing the likelihood of weakening root dentin via the recommended endodontic therapy.

Irrigation Protocol

McComb was the first to describe the smear layer that is formed during endodontic instrumentation. This layer is comprised of inorganic mineralized particles and organic tissues including necrotic and vital pulp tissue, odontoblastic processes, and bacteria (McComb and Smith 1975). The various components make up a friable and loosely adherent layer visible during and after canal preparation. The superficial layer is an amorphous non-granular layer that is approximately 1-2μm thick. A deeper second layer penetrates dentinal tubules in varying densities and depths up to 40μm. (Mader, et al., 1984).

Smear layer removal is important in endodontics, but has specific implications in the treatment of external inflammatory resorption. The smear layer contains bacteria, and can be considered a possible substrate for continued bacterial growth and penetration into the dentinal tubules (McComb and Smith 1975; George, et al., 2005). Bacteria may be found deep in dentinal tubules prior to treatment (Bystrom and Sundqvist 1985; Sen, et al., 1995), and presence of smear layer can block effective application of both irrigants

and medicaments used to target these pathogens (McComb and Smith 1975; Yamada, et al., 1983). It has been demonstrated by several studies that smear layer removal facilitates more efficient diffusion of hydroxyl ions through dentinal tubules for therapeutic effect when CH is placed as a medicament in the root canal system (Foster, et al. 1993; Saif, et al. 2008).

Sodium hypochlorite is the most widely used irrigant in endodontics. It effectively dissolves vital and necrotic pulpal tissue. It is a potent antimicrobial agent, killing most bacteria on contact (Bystrom and Sundqvist 1985; Wong and Cheung 2014), including disruption of biofilms (Hand, et al. 1978, Clegg, et al. 2006). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent commonly used to help facilitate smear layer removal. This irrigant removes the inorganic portions of the smear layer by chelating calcium ions. When used in tandem with NaOCl smear layer can be completely removed (Baumgartner and Mader 1987).

Yamada demonstrated complete smear layer removal with high volumes of hypochlorite and EDTA. His *in vitro* model used 10mL of EDTA followed by 10mL 5.25% NaOCl delivered into the canal as a final rinse to successfully remove smear layer (Yamada 1983). Calt demonstrated that the contact time of the irrigants used for smear layer removal has a large impact on dentin condition. He found a 10-minute application of 10mL EDTA caused excessive peritubular erosion, and suggests limiting the time exposure of EDTA during final rinse to 1 minute (Calt and Serper 2002). In 2005 Crumpton demonstrated that reduced volumes of EDTA are effective for smear layer removal with adequate time exposure, advocating a 1mL final rinse with EDTA over 1 minute (Crumpton, et al., 2005). Both volume and contact time remain important variables to smear layer removal with EDTA. Saito demonstrated that 1mL for 1 min provided adequate smear layer removal, but shorter time periods did not remove the entire smear layer (Saito, et al. 2008).

As previously mentioned, smear layer removal does affect diffusion of intracanal medicaments. After smear layer removal, dentinal tubules are left open and demonstrate a capillary type action (Stevens, et al 2006). Pashley found that smear layer removal increases dentin permeability nearly 25% (Pashley 1988). Foster was the first to study

the diffusion of calcium and hydroxyl ions through root dentin after using high volumes of NaOCl and EDTA to accomplish smear layer removal (Foster, et al., 1993). Nerwich was able to demonstrate similar diffusion patterns using a 1mL volume of EDTA for 5 minutes of contact time (Nerwich, et al., 1993). In 2008, Saif found that 3mL of EDTA with a final rinse of 10mL NaOCl was more effective in promoting hydroxyl ion diffusion to the external root surface than 1mL and equally effective to 10 mL EDTA (Saif, et al., 2008).

Surface Tension

In order to be maximally effective CH medicaments need to be densely distributed through the canal system in contact with dentinal walls (Sigurdsson 1992; Chamberlain 2009). After dissociation into respective Ca^{2+} and OH^- ions, particles diffuse through dentinal tubules to exert their biologic effects at the external root surface (Tronstad, et al., 1981; Esberard, et al., 1996). Obrien and Rosales have discussed that wettability may be of primary importance to penetration of solutions into dentinal tubules, when used as irrigants in the root canal system (O'brien, et al., 1997). Modification of surface tension is one strategy to increase wettability (Abou-Rass and Patonai 1982). Surface tension is a condition of intramolecular attraction at the surface of a liquid in contact with a solid tending to pull the molecules inward from that surface (Abou-Rass and Patonai 1982). When intermolecular bonds are decreased or destroyed, surface tension is decreased. Surfactants have been used to decrease surface tension of irrigants introduced into the root canal system (Stojocik et al 2010). Baker demonstrated an increased effect on antimicrobial properties when a surfactant was added to CH medicaments (Baker, et al. 2004).

Cunningham added ethanol to NaOCl in order to decrease surface tension of the liquid and ultimately to increase wettability of dentin (Cunningham, et al., 1982). Wettability refers to the ability of a liquid to maintain contact with a solid surface, resulting from intermolecular interactions when the two are brought together. The degree of wetting is determined by a force balance between adhesive and cohesive forces.

Stevens explored the idea of using alcohol as an agent to increase wetting of the dentin surface before sealer application. He found a final rinse of 95% ethyl alcohol prior to obturation increased sealer penetration by a significant margin over NaOCl alone (Stevens, et al., 2006). Final rinse with 95% ethyl alcohol may have a similar effect when used before application of CH intracanal medicaments. If calcium hydroxide is better able to contact dentinal surfaces or penetrate dentinal tubules this may increase the rate or magnitude of pH change noted at the external root surface after their application.

Specific Aims

1. To assess the rate and magnitude of pH change at the external root surface after placement of CH medicament or BC sealer when applying a final rinse of 95% ethyl alcohol.
2. To compare two commonly used premixed calcium hydroxide medicaments Ultracal XS and Calasept Plus and their ability to change pH at the external root surface.
3. To assess the ability of Bioceramic sealer to affect change in pH at the external root surface.
4. To assess the effect of remaining dentin thickness of pH measurement within the *in vitro* model.

Null Hypotheses

1. A final rinse with 95% ethyl alcohol after smear layer removal will not affect the rate or magnitude of pH change noted at the external root surface after placing medicaments including Ultracal XS, Calasept Plus, or obturation with EndoSequence Bioceramic Sealer.
2. There will be no difference between Ultracal XS and Calasept Plus in their ability to affect the magnitude or rate of pH change measured at the external root surface.
3. EndoSequence bioceramic sealer will not cause a change of pH at the external root surface when compared to negative controls.
4. Remaining dentin thickness will have not effect on pH measured at the external root surface in this *in vitro* model.

METHODS AND MATERIALS

The University of Minnesota Institutional Review Board: Human Subjects Committee determined that this study (1605E88142) was exempt from review. Approximately 150 single rooted anterior and premolar teeth were collected from various clinics including the University of Minnesota oral surgery clinic, the VA Hospital Minneapolis, MN, and the Union Gospel Mission dental clinic St Paul, MN. All teeth were stored in a solution of 0.1% Thymol in 0.9% Normal Saline until use in the study. 0.1% Thymol was used as a storage solution based on historic study demonstrating minimal affect on dentin permeability (Goodis, et al., 1991; Goodis, et al.,1993) and previous studies completed using this storage material (Haenni, et al., 2003).

Sample Selection:

One hundred single rooted teeth were selected for use in this study from the storage containers used for tooth collection. Teeth were selected based on morphology (single rooted: maxillary incisors, canines, or premolars, and mandibular canines or premolars), minimum root length requirement (15mm), and absence of root caries. Teeth were stored in a new container containing 0.9% Sodium Chloride (Baxter, Deerfield, IL) after selection. No preoperative radiographs were taken to avoid selection bias based on canal morphology. Teeth were examined using a Global G6 surgical operating microscope (SOM) at 5.1X magnification (Global Surgical Co, St Louis, MO) to eliminate teeth with defects including cracks, cementum defects, lateral canals that may affect the study model. This visual examination was repeated a second time after completion of cleaning and shaping protocol.

Cleaning and Shaping: *(Completed by two operators CS and JG)*

Selected teeth were soaked in 5.25% NaOCl for 30min to facilitate removal of soft tissue debris and periodontal ligament (PDL) (Foster, et al., 1993). Teeth were then decoronated using #557 carbide bur (Brasseler USA, Savannah GA) to achieve a 15mm standardized root length (Figure 1). The same bur was used to help facilitate access to root canal space if not readily available to #10 FlexoFile after decoronation (Dentsply,

Sirona, New York, NY). Teeth were discarded if the canal could not be negotiated with a #8 Flexofile (Dentsply, Sirona, New York, NY) after access. Roots were stored in a sterile specimen container for 24 hours in 0.9% Sodium Chloride after sectioning.

Following 24 hours of storage in sterile saline, teeth were instrumented by two operators using the ProTaper Gold file system (Dentsply, Sirona, New York, NY).

Figure 1. Example of WL determination with #10 flexofile

Root lengths were standardized to 15mm. A #10 FlexoFile was placed into the canal until it could be seen at the constriction using the SOM at 5.1 X magnification (Global Surgical Co, St Louis MO) (Figure 1). Glide path was refined to size #15 FlexoFile. A corrected working length (CWL) of 14.5mm was established for each root. The ProTaper Gold file system (Dentsply, Sirona, New York, NY) was then used per manufacturer's instructions with a final preparation size of F4 (40.06 – variable taper) at CWL. Irrigation during instrumentation was limited to 0.9% NaCl to avoid variation in irrigant volumes used in each sample preparation.

After preparation of the canal space, two cemental defects were created in each root under 8X magnification using the SOM (Global Surgical Co, St Louis MO). A #6 round bur, (Brasseler US, Savannah GA) 1.8mm in diameter, was used to create defects on the facial and lingual root surfaces at the midpoint of the root. Defects were made approximately 4mm in an occlusal and apical dimension with a depth sufficient to remove cementum (Foster, et al., 1993). Smear layer was then removed from the defects

by applying 17% EDTA with a microbrush (Microbrush, Grafton WI) for 1 minute followed by a rinse with distilled water (Saif, et al., 2008). During this phase, teeth were evaluated a second time for cracks, lateral canals or cemental defects that might compromise the study design.

Randomization:

During the preparation process approximately 8 teeth were discarded for various reasons including: unable to access canal, unable to instrument canal space per prescribed protocol, root fracture, and presence of root caries. The first 80 of the remaining prepared root segments removed from the specimen vial were selected for inclusion in the experiment.

A single 1 liter bottle of 0.9% NaCl was used to fill 160 4mL Shorty Lab Vials with rubber lined black plastic caps (Wheaton Industries, Tulsa, OK) with 2mL sterile saline. The bottle was thoroughly agitated before dispensing liquid to ensure uniformity of storage media. The first 80 vials were labeled numerically #1-#80. Individual teeth were removed from the storage container and placed 1 root per vial until all 80 vials were filled.

Using a randomization table generated in Excel (Microsoft Corporation Redmond, WA) teeth stored in the labeled vials were assigned to one of the 8 experimental groups: (see asterisk for explanation of 10 final groups in experiment)

EXPERIMENTAL GROUPS

Group 1	(n=10, 1-10):	Ultracal XS (EtOH+)	(UC+)
Group 2	(n=10, 11-20):	Ultracal XS(EtOH -)	(UC-)
Group 3	(n=10, 21-30):	Calasept Plus (EtOH +)	(CS+)
Group 4	(n=10, 31-40):	Calasept Plus (EtOH -)	(CS-)
Group 5	(n=10, 41-50):	BC sealer (EtOH +)	(BC+)
Group 6	(n=10, 51-60):	BC sealer (EtOH -)	(BC-)
Group 7	(n=6, 61-66):	Negative Control (EtOH +)	(Neg +)
Group 8	(n=6, 71-76):	Negative Control (EtOH -)	(Neg -)
Group 9	(n=7, 81-85, 70, 80):	Saline	(Saline)
Group 10	(n=6, 67-69, 77-79):	Full Wax	(Full Wax)

*** In groups 7 and 8 roots randomized to vials labeled 67, 68 ,69 and 77, 78, 79 were selected as technique controls. These 6 samples make up a second negative control group labeled "Full Wax" Group 10. These root were prepared in tandem with their respective treatment groups 67=1, 68=3, 69=5, 77=2, 78=4, 79=6 but were sealed completely in wax.*

*** Vials 70 and 80 were dedicated as saline only and were included with an additional 5 samples that were filled at time of randomization (Group 9 "Saline").*

After randomization, vials were relabeled to reflect the correct experimental group and given new numeric labels 1-80 correlating to their respective groups before sample preparation. Roots were not transferred to the second set of vials until sample preparation was completed and samples were sealed with wax.

Preparation – Final: (completed by 2 operators CS and JG)

Before randomization and after preparation of root defects, all roots were irrigated with 3mL of 5.25% NaOCl using a 30 ga Max-i-Probe side vented irrigation needle (Dentsply, Sirona, New York, NY) placed 1mm short of working length over 1 min. After randomization, each group was irrigated separately. All samples in each group received 1mL 17% EDTA using a 30 ga Max-i-Probe side vented irrigation needle

(Dentsply, Sirona, New York, NY) placed 1mm short of working length over 1 min followed by 3mL 5.25% NaOCl over 1 min as previously described in order to complete smear layer removal (Saif, et al., 2008).

Groups 1, 3, 5, 7 (EtOH + groups) received a final rinse of 1mL 95% ethyl alcohol delivered using the same side vented Max-i-probe needle, while Groups 2, 4, 6, 8 (EtOH – groups) were considered complete after the final rinse of 3mL 5.25% NaOCl. Canals were dried with paper points (Dentsply, Sirona, New York, NY) after irrigation was completed.

The techniques used to place both UltraCal XS (Ultradent Products, South Jordan UT) for groups 1 and 2 and Calasept Plus (Dentsply, Sirona, New York, NY) for groups 3 and 4 were the same. Medicaments were delivered from a premixed syringe using a 25mm 30ga Navitip syringe (Ultradent Products Inc, South Jordan UT). A small amount of medicament was expressed from the Navitip before placement into the canal to ensure adequate flow. Needles were inserted to WL and then retracted approximately 1mm until no longer binding. Medicament was slowly expressed until the canal space was filled (Staehle, et al.,1997) (Figure 2). Radiographs were taken to ensure adequate density of medicament placement (Simcock and Hicks 2006). Any excess was carefully removed from apical or coronal aspects of the root. A small increment of Cavit-G (3M ESPE, Minneapolis, MN) was placed coronally to form an initial barrier before sealing with wax.

Groups 5 and 6 were obturated with EndoSequence BC sealer. Following irrigation, all 20 roots from the two groups were filled with the BC sealer using the plastic delivery tip provided by the manufacturer (Figure 2). Following sealer delivery, prefit F4 gutta-percha (GP) cones from (Dentsply, Sirona, New York, NY) were dipped in excess sealer and were placed to WL. The GP was seared at the orifice with a System B heat plugger (Kerr Corporation, Orange, CA) and condensed with an endodontic plugger. Excess sealer was delicately removed from the apex or coronal aspect of the root prior to sealing samples. No Cavit-G was used for initial seal in this group.

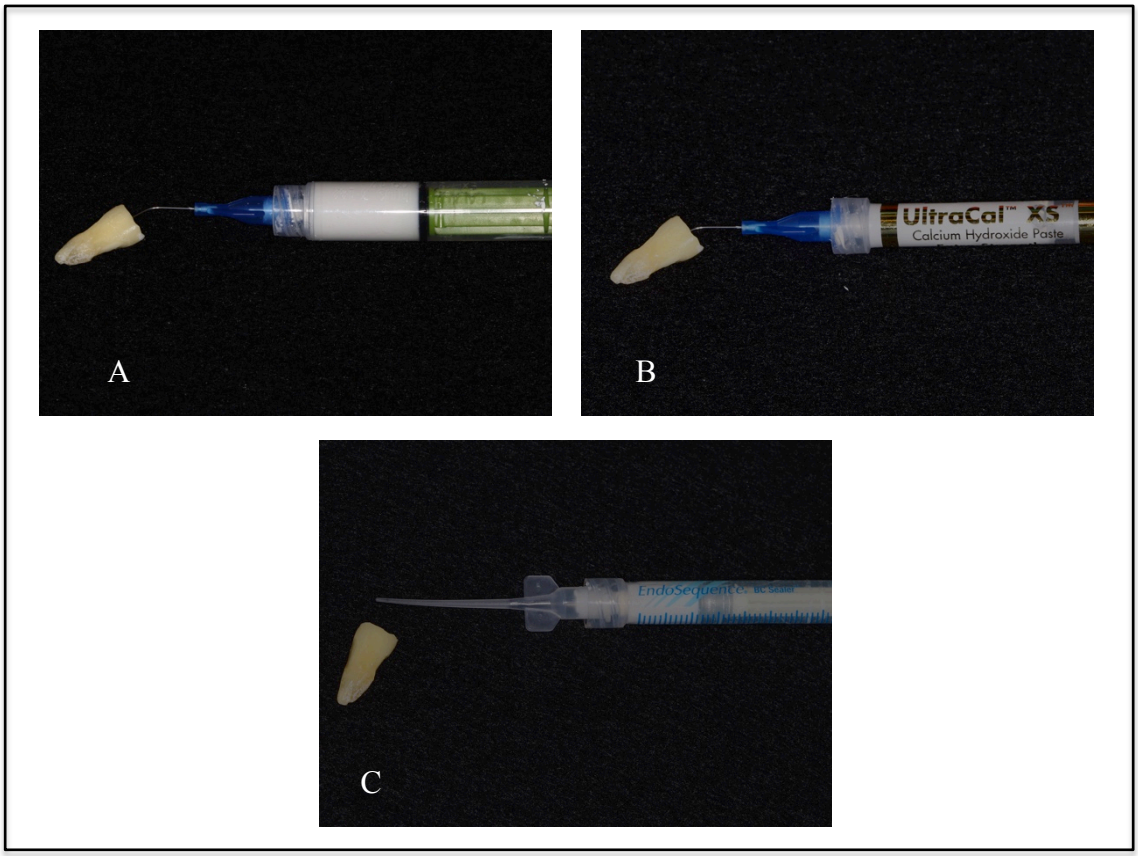


Figure 2. Application of CH pastes and BC sealer. (A) - Calasept Plus. (B) – UltraCal XS (C) - EndoSequence BC sealer.

After placement of medicament or sealer, roots from each group were sealed at apical and coronal ends using two layers of heated sticky wax (Kerr Co., Orange CA). Approximately 3mm of coronal and apical tooth structure was sealed at each end, taking care not to place wax over the defects (Figure 3, 4). Controls #67, 68, 69, 77, 78, 79 were completely covered in two layers of wax after preparation with their respective groups. After wax had set (approximately 45 seconds) tooth roots were rinsed with distilled water, blotted dried and then placed in their newly labeled lab vials filled with 2mL sterile saline (Wheaton Industries, Tulsa OK) (Figure 3).

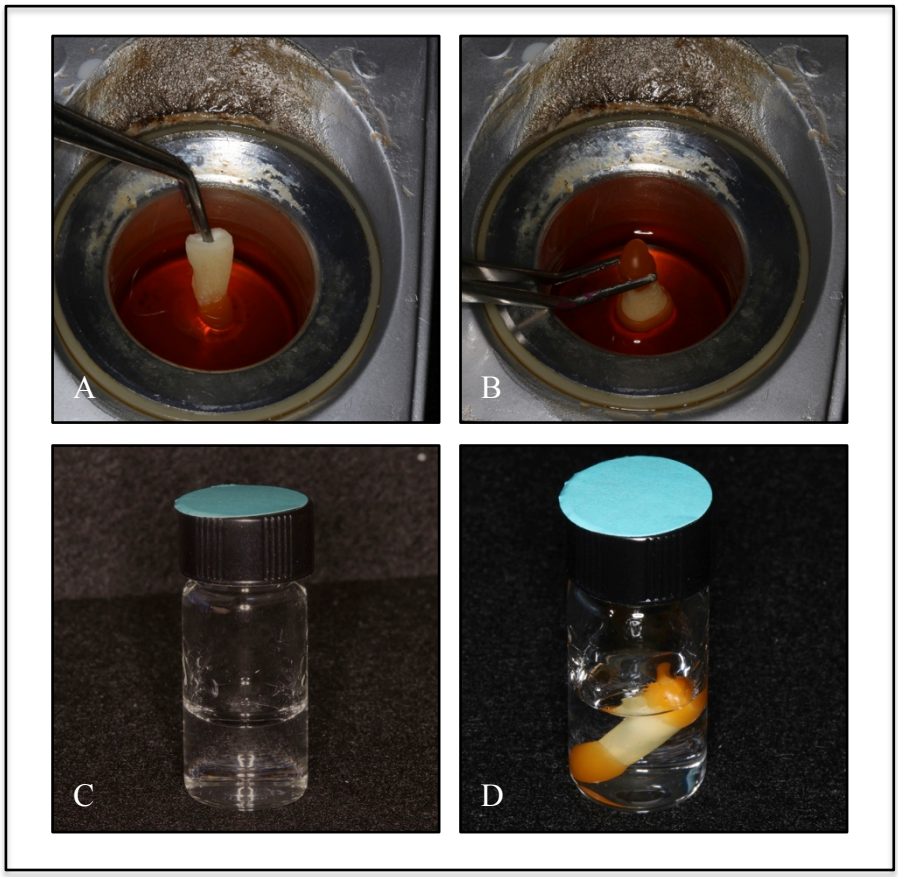


Figure 3. (A/B) sealing of apical and coronal segments with heated sticky wax. (C) Vial prefilled filled with 2.0mL saline. (D) Sample placed in saline after wax and final rinse.

Figure 4. Close up of root samples after covering with wax. Defects are visible from F and proximal view

Sample pH was measured at the following time intervals: 0, 1, 2, 4, 7, 9, 12, 16, 19, 22, 28 days based on previous studies (Chamberlain, et al., 2009; Saif, et al., 2008; Nerwich, et al., 1993). A PerpHect Ross Micro Combination pH Electrode model 8220BNWP (Thermo Scientific, Minneapolis, MN) with Orion 3 Star bench top pH meter (Thermo Electron Co, Waltham MA) was used to make measurements. The electrode was calibrated prior to each session using stock pH buffers 4.0, 7.0, and 10.00 (Thermoworks, American Fork, UT) (Figure 5). Prior to measuring, each sample was agitated by hand for approximately 5 seconds. The microelectrode was submerged in the saline surrounding the root and measurements were recorded after stabilization in the Autoread function (Figure 5). The electrode was blotted dry with a Kimwipe (Kimberly-Clark, Roswell, GA) between each sample per group (Saif, et al., 2008). The electrode was then rinsed with distilled deionized water and dried with a Kimwipe (Kimberly-

Clark, Roswell, GA) before measuring the next group. Between groups, pH 7.0 buffer was measured to ensure accuracy of meter readings.

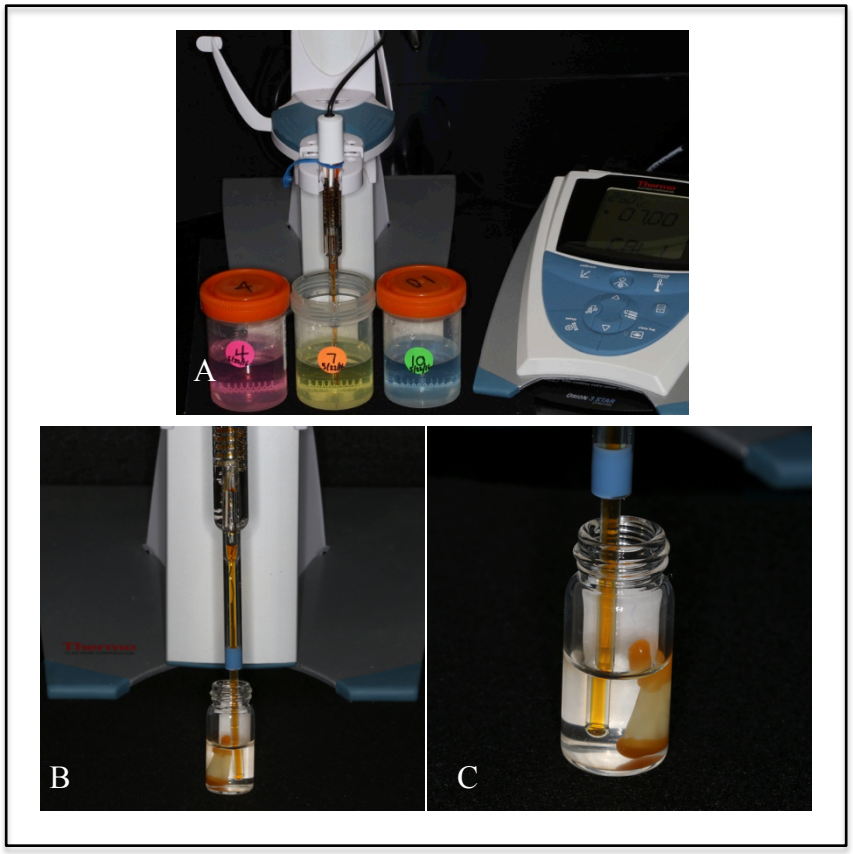


Figure 5. (A) Calibration with 3 premixed buffer solutions. (B) Sample pH measurement with microelectrode. (C) Close up of microelectrode in solution.

Defect Measurement:

After conclusion of the study teeth were removed from their respective vials and re-examined at 8X magnification using the SOM (Global Co., St. Louis, MO). The status of the coronal and apical seals were assessed and recorded. The roots were then radiographed in M-D direction to demonstrate no changes in volume of medicament over the time of the study. After radiograph, roots were sectioned using a diamond-coated disc in a slow speed straight handpiece (Brasseler USA, Savannah GA). Sectioning was completed at the midpoint of root defects and four measurements were made to determine

the average distance from canal wall to created external defect. The buccal and lingual defects were measured in both the coronal and apical segments using an electronic caliper (S-T Industries Inc., St James, MN) under 8 X magnification (Global Surgical Co., St Louis MO) (Figure 6). The mean was used to correlate dentinal thickness to pH change during statistical calculation.

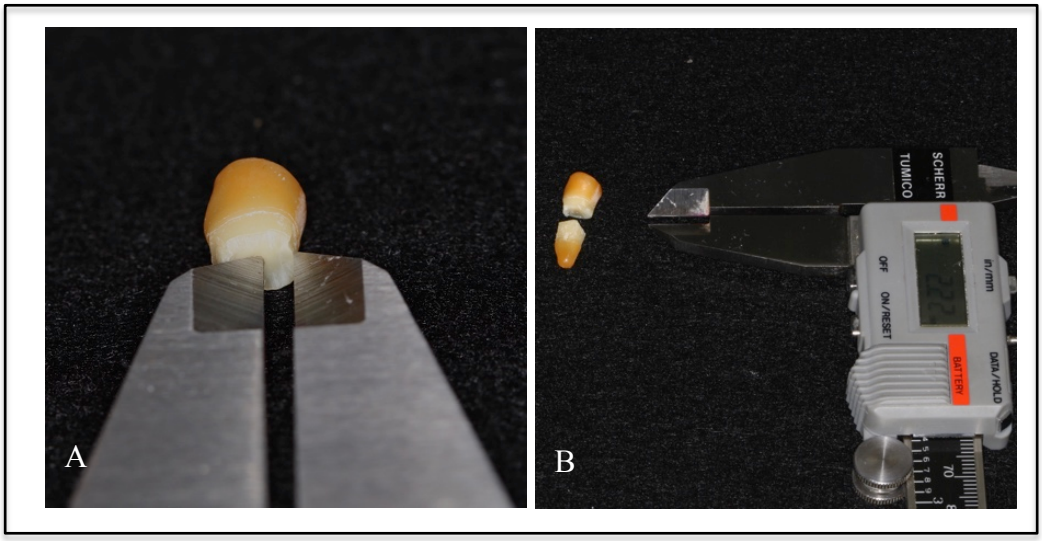


Figure 6. (A). Close up of measurement from canal wall to external surface at midpoint of defect. (B) electronic caliper and sectioned root halves.

STATISTICAL ANALYSIS

Results are from a multiple linear regression model with medicament (including the presence of no medicament), presence of EtOH, and their interaction as independent variables. A separate model was run for each time point. pH was the outcome variable. SAS V9.3 was used for the analysis. A two-group t-test was used to compare maximum change in pH between groups from time 0 to maximum mean pH value within each group. A separate multiple linear regression model was used to correlate pH to dentinal thickness at each time point using SAS V9.3.

RESULTS

Mean pH, standard error, and average remaining dentin thickness (RDT) values are listed for the eight experimental groups included in the statistical analysis (Table 1). Mean pH values at each time point are graphically represented in Figure 7. Group 9 (saline) and Group 10 (full wax NC) were included to validate experimental protocol but were not included in statistical analysis. pH values of group 10 (full wax NC) are presented in Table 2. It is important to note that sample #77 did not maintain a neutral pH as expected. This raises questions about the integrity of the wax seal used to prevent hydroxyl ion diffusion through apical foramen or coronal access. A graphic representation of the full wax control group can be seen in Figure 8 demonstrating the failure of the wax seal in sample 77.

Failure of the full wax negative control led to further observation of samples to explore possible reasons for failure of the wax seal, and the extent to which this may have affected the experimental model. A visual inspection of all samples using the SOM was completed after day 28. A total of 15 samples were identified with suspected compromise of either apical or coronal wax seal under 8X magnification (Table 3). pH values from each experimental group were evaluated, and seven samples with outlying final pH values were identified. Only two of the samples in the experimental groups with outlying pH values at day 28 were found to have poor quality coronal or apical seals (sample 4 and 28 seen in Table 3), demonstrating a poor correlation between visual identification of wax seal compromise and pH values. Sample 15 was found to have

cementum damage that was not noted prior to randomization. Table three lists all samples with poor quality seals and high pH values as well as RDT values of these samples.

In attempt to account for outlying samples, a sensitivity analysis was completed removing the 6 outlying data points with highest pH values at day 28. (#77 was not included in original statistical analysis). Samples were selected visually as outliers from their groups. All but one of the selected sample were 2 SD's outside the mean for their respective groups. These samples were chosen to more conservatively identify the possible effects of medicament and EtOH on pH change measured in this experimental model. The sensitivity analysis was compared to results from the original data set. Differences were noted between the two analyses, indicating the outlying data points significantly affected statistical analysis (Table 4, 5, 6).

Results from the original data set and sensitivity analysis will both be presented. Primarily general data trends will be considered rather than inferring statistical significance from either model. Interactions with p-values < 0.05 are represented by a red "x" in Tables 4, 5, and 6 while those >0.05 were represented with a black "o". Both the initial and sensitivity analysis are reported.

The effects of medicament, ethanol, interaction between ethanol and medicament, as well as effects of RDT were the initial metrics tested via a multiple linear regression model (Table 4). There is a significant medicament effect identified on days 0, 1, 2, 12, 16, 19, 22, 28 in both the original and sensitivity analysis (Table 4) demonstrating good agreement. This is a general effect of medicaments indicating there are differences that exist within the three medicament groups and their control groups that can be attributed to medicament used. Further pairwise comparisons were useful in helping to identify specific differences between groups (Appendix VII). The BC sealer group demonstrated the highest initiatl pH values. At day 12 the pH of CS+ and CS- groups were significantly higher than all other experimental groups as demonstrated by the sensitivity analysis (Appendix VII).

At days 1 and 2 a significant ethanol effect was identified by the variable regression model in the original data set. The sensitivity analysis demonstrated greater effect of the

ethanol variable with p values <0.05 noted on days 1, 2, 4, 12, 16, 18, and 22 (Table 4). Further comparison within EtOH + and EtOH - groups isolated this effect in the original analysis to the CS+ group only on days 0, 1, 2, 4. The sensitivity analysis revealed significant differences on days 0 and 1 in the CS + group and days 1 and 4 in the UC + group. All groups demonstrated differences between EtOH positive and EtOH neg groups between days 12, 28 (see Table 5 for specific results). These trends are graphically represented in figures 9b and 10b with a relative spike in pH from days 0-1 and rise in pH different from their EtOH- counterparts in both the UC+ and CS+ groups.

Interaction between medicament variable and the ethanol variable was tested at each time point in the regression analysis. Conflicting results were found between original and sensitivity analyses (Table 4). Days 16-28 were identified as a having significant interaction only in the sensitivity analysis. The implication of this interaction is that the effect of ethanol cannot be generalized to all medicaments at that time point, but instead should be considered on a group by group basis. This means findings from a specific group and time point cannot be applied to other experimental groups or time points and vice versa.

RDT at the defect site was measured after conclusion of the experiment. This variable was added to the statistical model in order to demonstrate how RDT affects pH over time. There was a significant effect detected, relating decreasing dentin thickness to increasing pH values from days 4-28. The results of this analysis are reported in Figure 13. This effect is not confirmed as dramatically in the sensitivity analysis with significance only being noted at time points 1 and 4 (Table 5).

In terms of general trends noted in the data, both CH medicament groups demonstrated a rise in pH over the first 12 days followed by a decline to levels just above baseline of negative controls. Figure 9 represents the UC+ and UC- groups, while Figure 10 represents CS+ and CS- groups. The BC sealer groups demonstrated an initial pH higher than all experimental groups (see appendix VII). This was true in both original data set and sensitivity analysis. BC sealer groups experienced slow decrease in pH until day 12 after which pH dropped quickly to baseline levels or lower. The BC- group ended with the lowest pH value in the experimental model (Figure 11). Negative controls with

and without ethanol demonstrated a measured decline from an initial value of 7.63 (NC+) and 7.73 (NC-) to a final value of 6.40 (NC+) and 6.13 (NC-). The NC+ groups demonstrated a greater rate of pH decline from days 12-19 than the NC- groups, but both ended at a similar baseline level in this model (Figure 12). The original evaluation revealed no differences between NC+ and NC- groups while sensitivity analysis did find a difference between these two groups at days 12, and 16. Although no statistics were applied to the saline control group it is important to note that minimal variation in pH values were noted over time. pH values remained in a consistent range just above 7.0. The maximum average pH of saline was 7.29 and mean pH did not decrease below 7.0.

The magnitude and direction of pH change in each group was graphically represented by plotting the difference in pH between time points in each group. Subtracting pH values between each successive time point allows for a better visual representation of pH effect. For example, the first value is calculated by subtracting pH at day 0 from day 1, and was labeled "0-1". It is important to recognize that time points vary in length and this is a difference model and is not based on slope, which would represent the pH over time. These representations are included in Figures 9, 10, and 11 below.

Table 1. Mean pH Values Measured at Each Time Point with SE. Associated mean RDT values.

DAYS		0	1	2	4	7	9	12	16	19	22	28	RDT
UC +	pH	7.15	7.48	7.40	7.42	7.54	7.50	7.20	7.08	7.15	7.19	7.09	2.46
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.23	0.25	0.26	0.27	
UC -	pH	7.38	7.35	7.26	7.33	7.64	7.81	7.50	7.34	7.41	7.51	7.54	2.28
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.24	0.26	0.26	0.28	
CS+	pH	7.18	7.93	7.79	7.81	7.89	7.97	8.22	7.56	7.16	7.23	7.09	2.40
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.23	0.26	0.26	0.28	
CS -	pH	7.62	7.54	7.40	7.29	7.47	7.74	8.08	7.77	7.64	7.59	7.51	2.74
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.24	0.26	0.26	0.28	
BC+	pH	8.32	7.99	7.70	7.62	7.61	7.65	7.73	6.89	6.77	6.75	6.68	2.45
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.23	0.25	0.26	0.27	
BC-	pH	8.12	7.81	7.63	7.47	7.42	7.40	7.43	6.72	6.10	5.98	5.82	2.52
	SE	0.14	0.09	0.10	0.12	0.16	0.19	0.19	0.23	0.25	0.26	0.27	
Neg +	pH	7.63	7.54	7.48	7.37	7.33	7.22	6.29	6.16	6.16	6.24	6.40	2.52
	SE	0.18	0.18	0.13	0.16	0.21	0.25	0.25	0.31	0.34	0.34	0.36	
Neg -	pH	7.73	7.59	7.49	7.35	7.25	7.16	6.98	6.68	6.41	6.41	6.31	2.81
	SE	0.18	0.18	0.13	0.15	0.21	0.25	0.24	0.30	0.33	0.33	0.35	
Saline	pH	7.19	7.14	7.03	7.22	7.29	7.24	7.15	7.10	7.01	7.10	7.00	N/A
	SE	0.08	0.06	0.03	0.04	0.03	0.05	0.04	0.03	0.01	0.05	0.06	

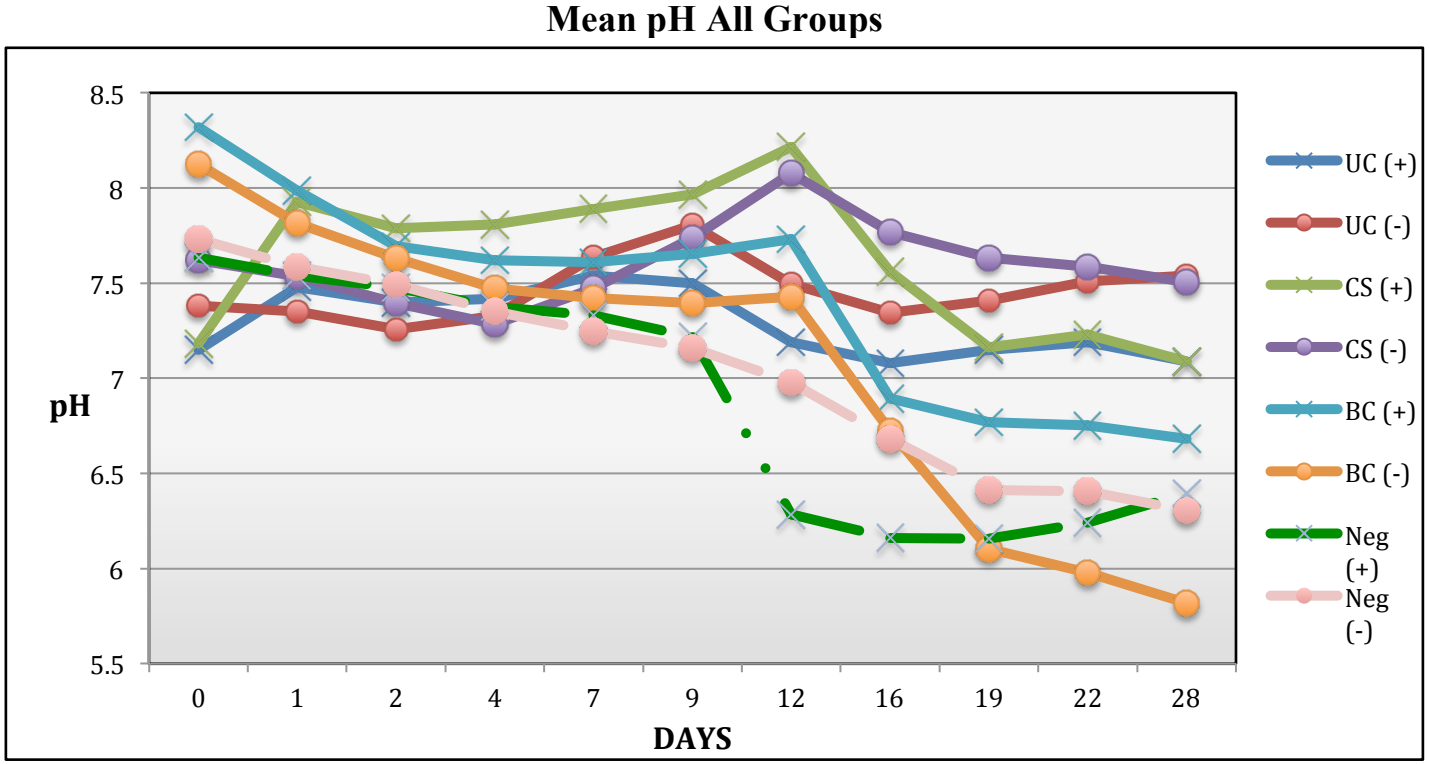


Figure 7. Average pH of all experimental groups plotted in line graph. (See legend for group labels)

Table 2. Group 10 - pH of full wax NC (n=6)

DAYS		0	1	2	4	7	9	12	16	19	22	28
UC+	67	7.73	7.54	7.38	7.04	7.09	7.33	6.60	6.47	6.39	6.47	6.28
CS+	68	7.01	6.86	6.82	6.66	6.99	6.91	6.75	6.74	6.65	6.94	6.30
BC +	69	7.14	7.12	7.03	7.04	7.26	7.13	6.62	6.46	6.38	6.60	6.42
UC-	77	7.65	7.52	7.35	8.06	9.64	10.12	10.81	11.09	11.14	11.13	11.22
CS-	78	7.08	7.27	7.19	7.42	7.52	7.39	7.09	6.83	7.00	7.22	7.03
BC -	79	8.11	7.61	7.47	7.56	7.49	7.46	5.71	5.40	5.35	5.30	5.24
Ave		7.61	7.47	7.34	7.68	8.22	8.32	7.87	7.77	7.83	7.88	7.83

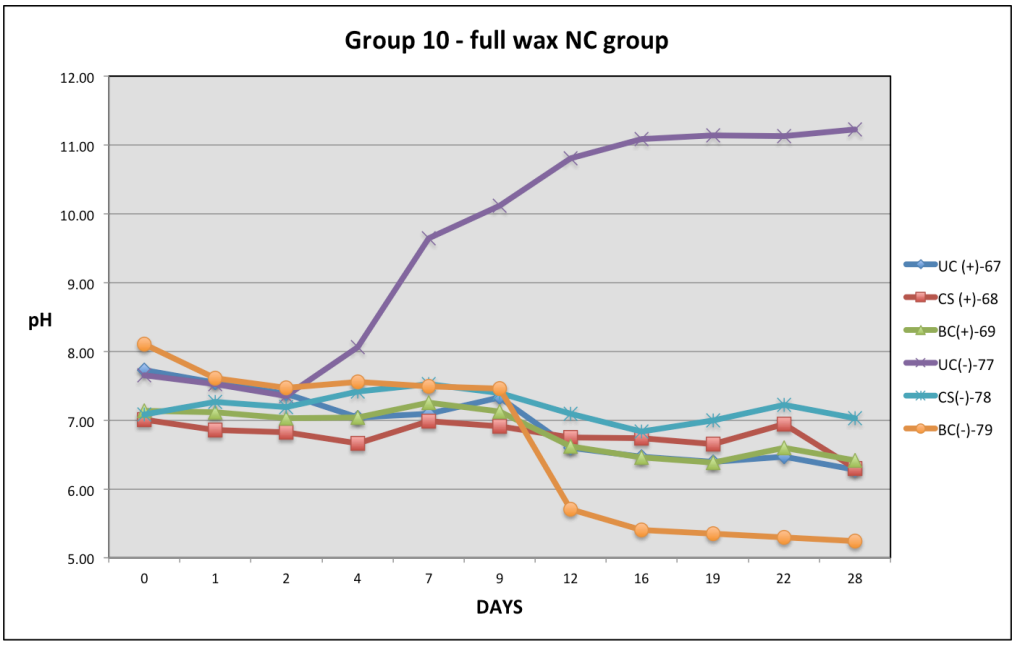


Figure 8. line graph of each full wax control. Sample #77 is failed neg control

Table 3. Comparison of samples with outlying pH values and samples with suspected defects of coronal or apical wax seal.

Groups	pH Outliers	RDT	ph Value	samples with compromised wax seal	RDT	Description
UC+	3	2.32	8.04	2	2.87	AS
	*4	1.71	8.22	*4	1.71	CS
				8	2.10	CS
UC -	15	2.00	10.78	15	2.00	**CD**
				16	2.38	AS
				18	2.40	AS
CS+				26	2.33	AS
	*28	1.99	10.69	*28	1.99	AS
CS-	40	2.70	10.08	32	2.43	CS
				36	3.00	AS
				38	3.17	CS
BC+	47	1.65	10.47	NONE		
BC-	NONE			NONE		
Full+	NONE			67	2.30	AS
Full-	*77	2.73	11.22	*77	2.73	CS,AS

*** Denotes samples that demonstrate both high pH and defect in wax seal. Only two samples fit these criteria.**

CS=Coronal Seal
AS=Apical Seal
CD=Cementum Damage

Table 4. Main Effects From Variable Linear Regression Model

Time	Med		EtOH		Interaction		RDT	
	1st	Sens	1st	Sens	1st	Sens	1st	Sens
0	x	x	o	o	o	o	o	o
1	x	x	x	x	o	o	o	x
2	x	x	o	x	o	o	o	o
4	o	o	x	x	o	o	x	x
7	o	o	o	o	o	o	x	o
9	o	o	o	o	o	o	x	o
12	x	x	o	x	o	o	x	o
16	x	x	o	x	o	o	x	o
19	x	x	o	x	o	x	x	o
22	x	x	o	x	o	x	x	o
28	x	x	o	o	x	x	x	o

x = p<0.050; o= p>0.050; 1st=all data in model; sens= sensitivity analysis

Table 5. Comparing Use of Ethanol Within Groups

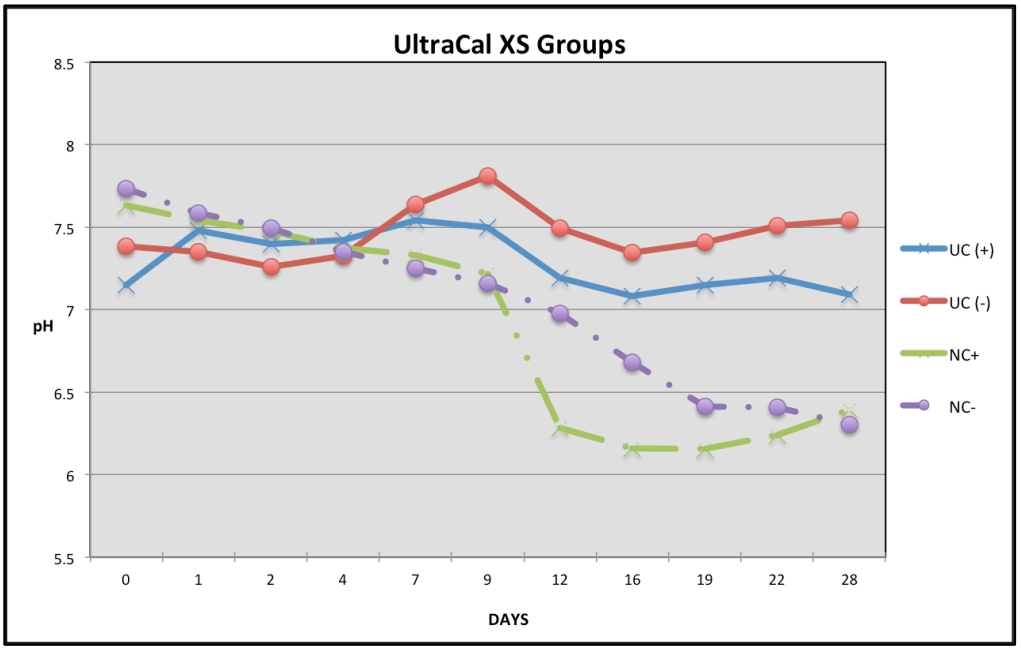
Time	BC		CS		NC		UC	
	1st	sens	1st	sens	1st	sens	1st	sens
0	o	o	x	x	o	o	o	o
1	o	o	x	x	o	o	o	x
2	o	o	x	o	o	o	o	o
4	o	o	x	o	o	o	o	x
7	o	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o	o
12	o	o	o	o	o	x	o	o
16	o	o	o	x	o	x	o	o
19	o	x	x	x	o	o	o	o
22	o	x	o	x	o	o	o	x
28	x	x	o	x	o	o	o	x

x = p<0.050; o= p>0.050; 1st=all data in model; sens= sensitivity analysis

Table 6. Comparing Effects of Medicament Used

Time	EtOH Positive BC+/ CS+/ UC+/ NC+		EtOH Negative BC-/ CS-/ UC-/ NC-	
	Original	Sensitivity	Original	Sensitivity
0	x	x	x	x
1	x	x	x	x
2	x	o	x	x
4	o	o	o	o
7	o	o	o	o
9	o	o	o	o
12	x	x	x	x
16	o	x	x	x
19	o	x	x	x
22	o	x	x	x
28	o	x	x	x

x = p<0.050; o= p>0.050



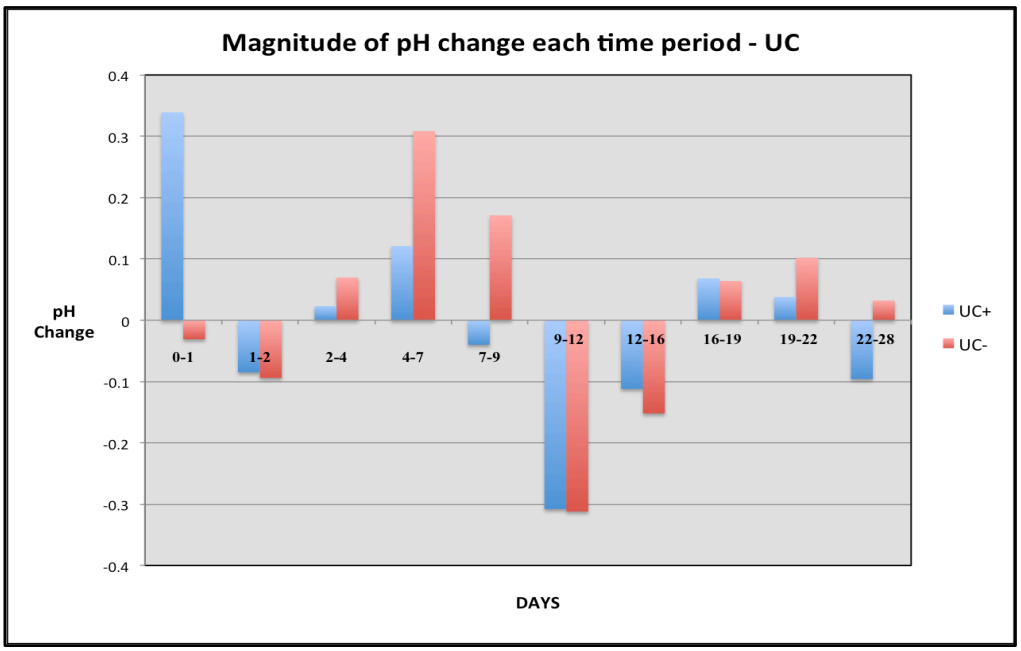
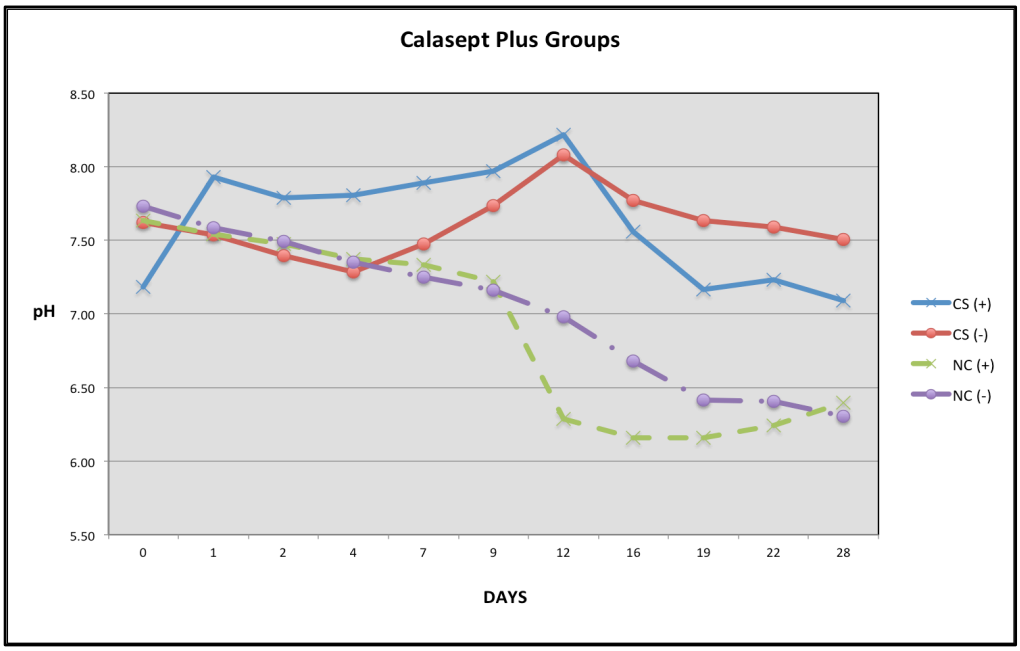


Figure 9a. Line Graph of average pH values at each time point for UC+/UC-/NC+/NC-
Figure 9b. Column graph demonstrating magnitude of pH at each time interval



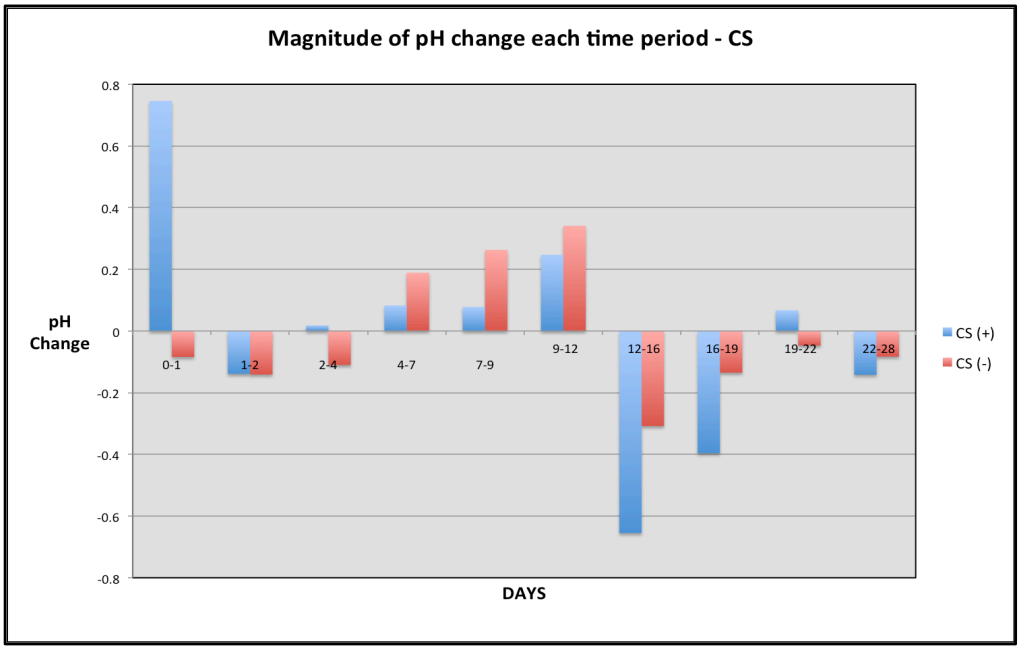


Figure 10a. Line Graph of average pH values at each time point for CS+/CS-/NC+/NC-
Figure 10b. Column graph demonstrating magnitude of pH at each time interval

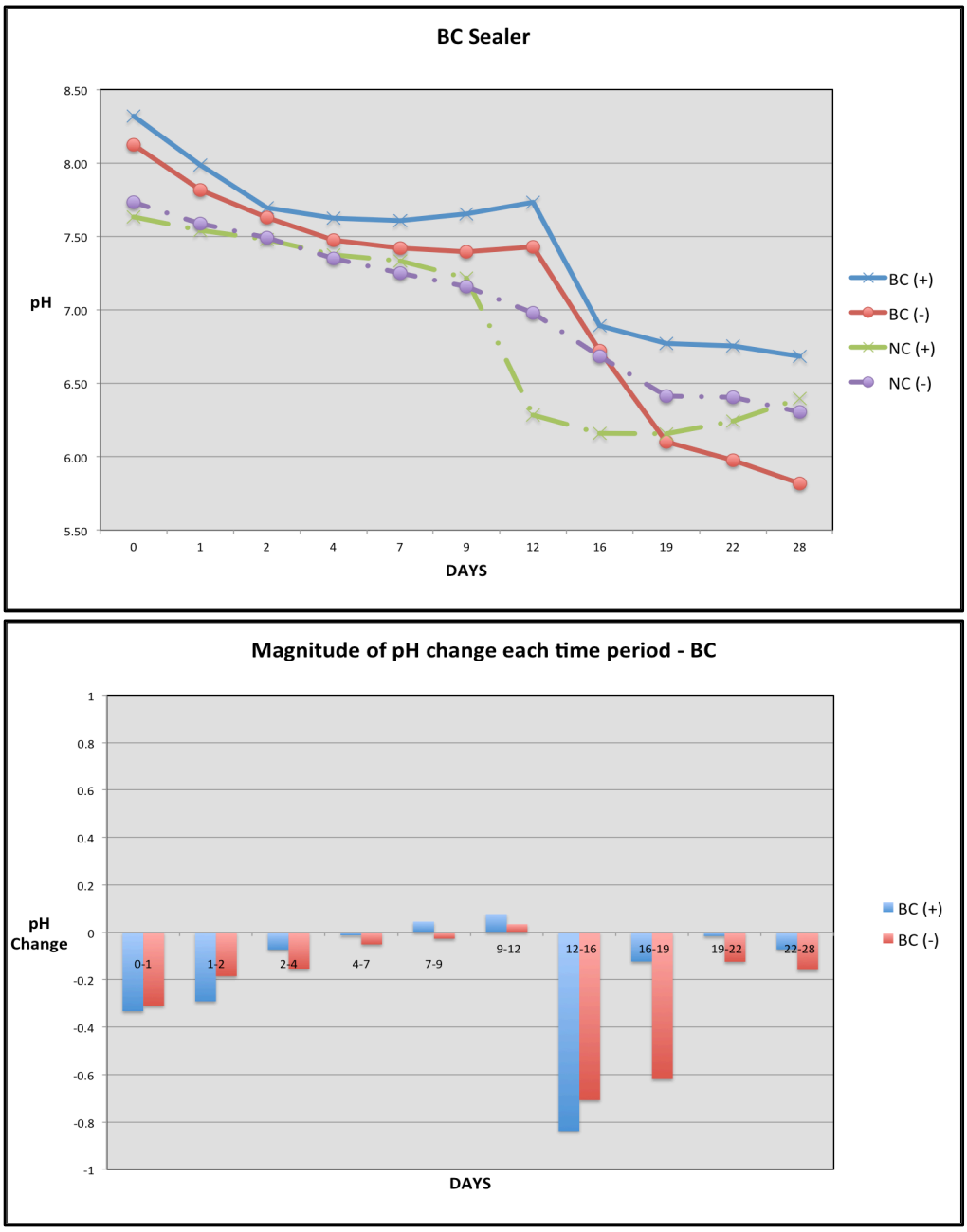
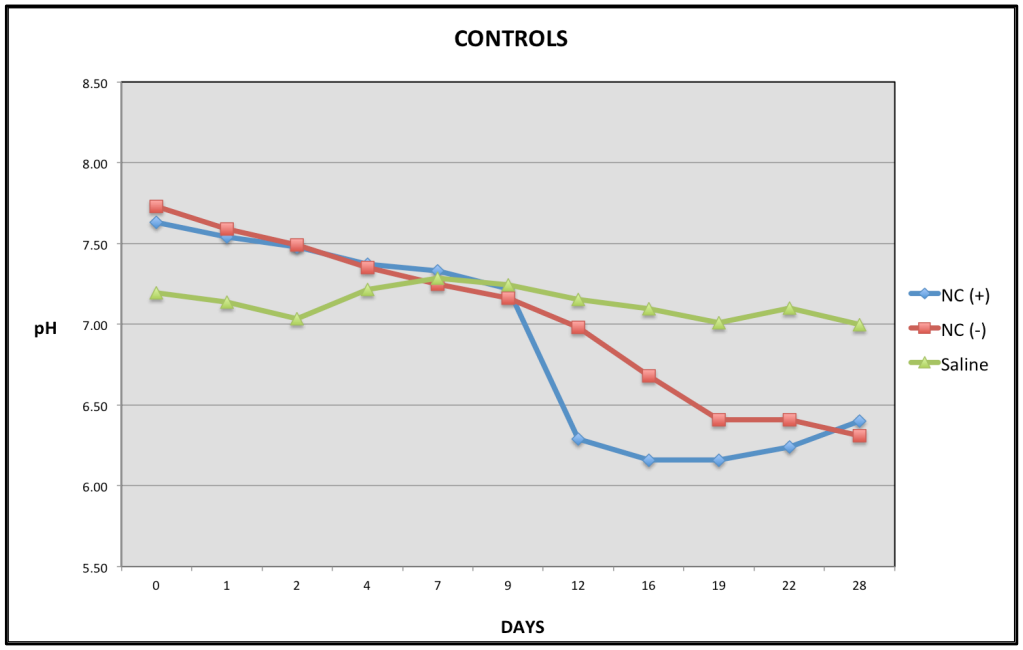


Figure 11a. Line Graph of average pH values at each time point for CS+/CS-/NC+/NC-
Figure 11b. Column graph demonstrating magnitude of pH at each time interval



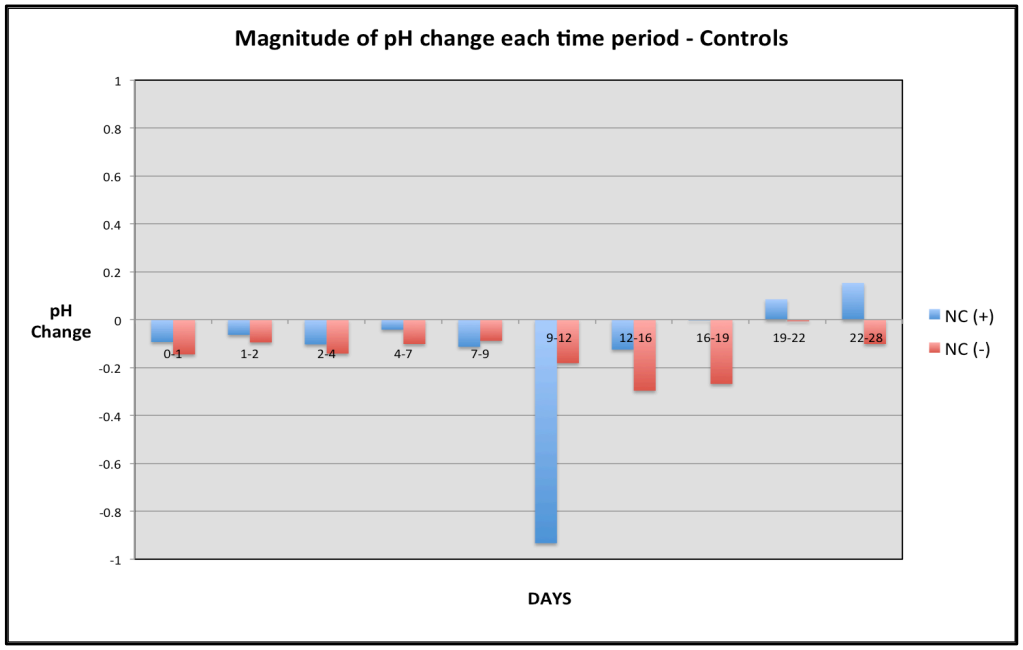


Figure 12a. Line Graph of average pH values at each time point for CS+/CS-/NC+/NC-
Figure 12b. Column graph demonstrating magnitude of pH at each time interval

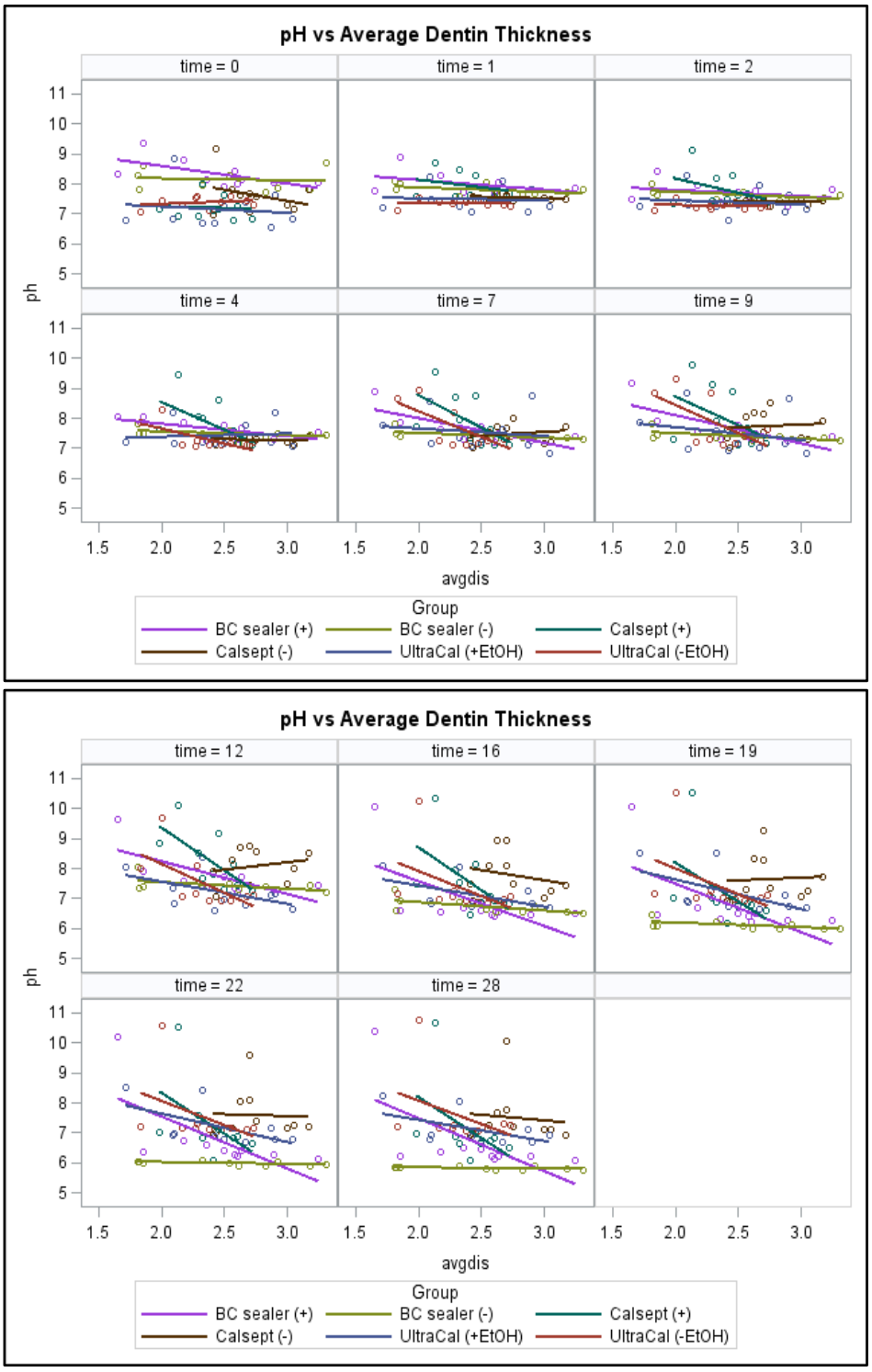


Figure 13. Line of best fit from each group demonstrating negative slope and correlation between remaining dentin thickness and pH.
When average distance was added as a covariate to the multiple linear regression model association between pH and average RDT was significant (p=0.0006)

Table 7. Minimum and Maximum pH values over 28 days

Mean pH	initial	Minimum	day	Maximum	day	Final
UC+	7.15	7.09	28	7.54	7	7.09
UC-	7.38	7.26	2	7.81	9	7.54
CS+	7.18	7.09	28	8.22	12	7.09
CS-	7.62	7.51	28	8.08	12	7.51
BC+	8.32	6.68	28	8.32	0	6.68
BC-	8.12	5.82	28	8.12	0	5.82
NC+	7.63	6.16	19	7.63	0	6.40
NC-	7.73	6.31	28	7.73	0	6.31
Full Wax	7.31	6.55	19	7.31	0	6.50

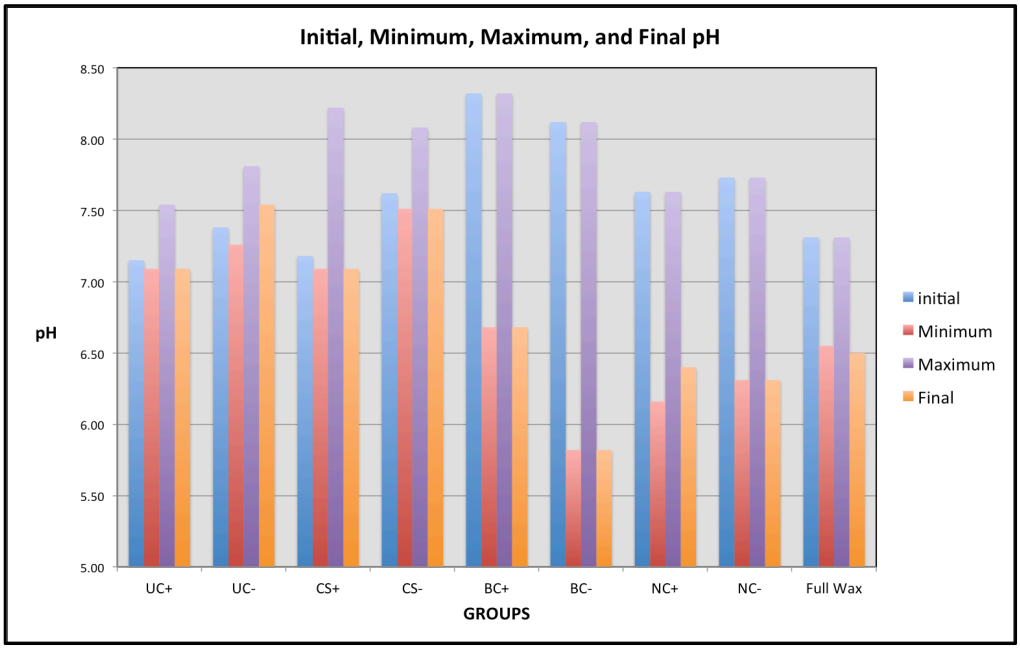


Figure 14. Initial, Minimum, Maximum, and Final pH values for each group

DISCUSSION

Several previous investigations have evaluated the effect of final irrigation protocols on the diffusion of CH through dentinal tubules. To date, no published research has evaluated the effect of a final rinse of 1mL 95% ethyl alcohol on this diffusion process. Only one study has looked at the use of BC sealer and its potential in affecting pH at the external root surface after obturation (Dudeja, et al., 2015).

In this investigation, ethanol was applied in a 1mL increment as a final rinse after smear layer removal in an attempt to increase the wettability of dentin (Cunningham, et al., 1998). A final rinse with ethyl alcohol can be linked to increased penetration of sealer into dentinal tubules during obturation (Stevens, et al 2008). Increasing the contact of CH medicaments or bioceramic sealers with the root canal wall and tubules may lead to more rapid and dramatic pH changes at the external root surface.

Movement of hydroxyl ions from canal space to the external root surface is thought to create a more alkaline environment that is favorable for osseous healing in cases of EIR (Tronstad, et al., 1981). Figures 9, 10, and 11 provide a visual representation of the mean pH values measured at each time point during the investigation for CH medicament groups, BC sealer groups, and their negative controls. Both Ultracal XS and Calasept Plus medicament groups demonstrated a nominal rise in pH above means of the negative control groups, demonstrating diffusion of hydroxyl ions through dentinal tubules. Peak pH levels occurred around day 12 with a maximum pH value of 8.22 demonstrated in the CS+ group (Figure 10 and 11). pH levels steadily declined over the remaining 14 days in both groups, while maintaining levels above the negative controls. These results are consistent with past experiments that have used a saline storage solution in order to measure pH at the external root surface (Foster, et al., 1993; Haenni, et al., 2003; Saif, et al., 2008). More recently, studies that have controlled for remaining dentin thickness have reported greater increases in pH values at the external root surface, possibly due to decreased remaining dentin thickness in these models (Javidi, et al., 2013; Ardensha, et al., 2002).

Several previous studies have failed to demonstrate any pH change at the external root surface after smear layer removal (Fuss, et al., 1989, Calt, et al., 1999). Buffering

capacity of dentin is one of the primary explanations for these past findings. Interaction between hydroxyl ions and dentin matrix proteins in the tubules can limit or prevent diffusion (Wang and Hume 1988). Deardorf noted that the chelating effect of EDTA exposed additional phosphate ions at dentinal surfaces, which caused a precipitation of calcium phosphate crystals after CH was applied. Precipitation of calcium phosphate crystal may act as a physical barrier to diffusion in dentin (Deardorf et al., 1994). With age, tubules become more mineralized and demonstrate a decrease in both size and number (Carrigan, et al. 1984). The age and character of dentin presents another physical barrier that may prevent successful medicament diffusion. These variables may all have had an effect on diffusion of hydroxyl ions in the current study model, contributing to rise in pH of smaller magnitude than found in previous investigations (Nerwich, et al., 1993; Ardensha, et al., 2002; Javidi, et al., 2013).

Ethanol was identified by the statistical model as having an affect at early time points in the CS+ groups (Table 4). The subsequent sensitivity analysis identified a similar pattern in the UC+ group (Table 5). In Figures 9-12, the change in pH between time points is plotted in a bar graph to demonstrate the direction and amplitude of pH change. In both the UC+ and CS+ groups, the majority of pH rise is noted during the first time increment. UC- and CS- groups demonstrate a delayed rise in pH with no substantial gains until day 4 (Figures 9 and 10). There appears to be a positive interaction between ethanol and CH in terms of the rate of pH change in both medicament groups. The effect of ethanol at later time points in the CS and UC groups may be due to maximal diffusion of $Ca(OH)_2$ at these time points.

The magnitude of pH change was measured in two ways. Maximum pH values from each group were compared directly between ethanol positive and negative groups. There was not a significant difference found between groups via this test (see pairwise comparisons in Appendix VII at time points lists in Table 7). The magnitude of change in pH was also recorded by subtracting the maximum pH value from time 0 in each group. Maximum pH change values were compared between groups irrigated with and without ethanol as a final rinse. No significant correlation in the magnitude of pH change

could be detected when making this comparison between groups, indicating no effect of ethanol on magnitude of pH change (Table 7, Figure 14).

The bioceramic sealer group demonstrated pH values above those of the negative control at the first two time points (Figure 11), which was considered "significant" in the BC+ group only (Appendix VII). This finding was consistent between original and sensitivity analyses. pH values were maintained at a level above the negative control until approximately day 12, after which pH levels dropped at a more dramatic rate. This trend of an initial alkaline pH that is maintained for about 1 week is consistent with similar models conducted with MTA as a comparison group (Hansen, et al., 2011, Heward, et al., 2011). The trend also compares favorably with Dudeja's findings over the first 12 days. The current model demonstrates a return to baseline levels that was not reported in other experiments. It is possible that use of GP during obturation may have decreased available CH for diffusion relative to the model used by Dudeja. Increase in sealer volume may explain the capacity to sustain alkaline pH for a longer time period (Dudeja, et al., 2015).

BC sealer has been demonstrated to penetrate dentinal tubules up to 200μm when used in either a single cone or warm vertical obturation technique (McMichael, et al. 2016). This material sets via a hydration reaction similar to mineral trioxide aggregate and will release calcium hydroxide during this time (Han et al., 2013). There is some controversy as to whether the BC sealer setting reaction behaves similarly in a dentin model compared to a bench top model. Xuereb reported that no CH formation could be detected during sealer set in a dentin fluid filtration model (Xuereb et al., 2015). Therefore, both bioactive properties and chemical property of increased alkalinity are called into question *in vivo*. In the current model, there appears to be some diffusion of hydroxyl ions based on the sustained alkaline pH over the first 12 days. This may indicate that BC sealer can produce CH during setting reactions conduced in a dentin model.

It has been reported that in the absence of moisture BC sealer is unable to set (Xuereb, et al., 2015). There is also great variability in setting time reported based on moisture content, with extension of final set up to 168 hours with excessive moisture

(Loushine, et al., 2011). No hardness testing was performed in this experiment to determine if sealer was set after termination of the experiment. However, visual and tactile investigation was completed after sectioning of teeth with a diamond disc. At the midroot level, BC sealer appeared to be set in all samples, including the full wax control samples.

The BC+ groups maintained a higher pH than the BC- group throughout the experiment. Differences between groups were significant only upon termination at day 28 in the original model and included days 19 and 22 as well in the sensitivity pairwise analysis (Apendix VII). It is possible that use of 95% ethyl alcohol as a final rinse allowed for greater penetration of sealer into dentinal tubules. This may have increased moisture availability in dentinal tubules nearer the external root surface where defects were present, and thus facilitated a more complete setting reaction. A more complete setting reaction could account for a greater volume of CH reaction product available for diffusion in the BC + group. Lower pH in the BC- group may be due to the decreased penetration of sealer. This may have kept sealer and CH produced further from the external root surface, thus decreasing diffusion to the external surface. The BC– group demonstrated the lowest pH in the experimental model with a mean value of 5.82 (Table 1). It is unknown if there are byproducts of the setting reaction of bioceramic sealer that may negatively impact alkalinity properties in a dentin model. Further investigation is required to clarify this finding. It appears that BC sealer's potential to affect pH at the external root surface may be limited to approximately 12 days when examining pH values in this *in vitro* model.

Considerations of experimental design:

Any time there are failures of the negative control in an experimental model, it is important to clarify why this may have occurred and what may be done in future investigations to improve upon the current model. The following discussion highlights several areas that were identified as weaknesses, and what may be improved going forward.

Negative Controls

Resins, waxes, and nail varnish have been used individually and in combination to seal teeth in similar experimental models. Heated sticky wax was chosen in this model because it was available, practical to apply after delivery of medicaments, and did not seem to affect pH change in a short duration pilot study. In a previous pilot, finger nail polish reacted poorly after irrigation of defects with 17% EDTA and was excluded from use. Sticky wax was also similar to the utility wax used by Foster, and was used in three of the most recent studies reviewed prior to initiation of this study (Hansen, et al., 2011; Ozdemir, et al., 2008; Dujeda, et al., 2015).

To date, no experimental models have included a negative control of their treatment group with a complete coating of wax to verify its sealing ability. Foster described a pilot study where utility wax was used to seal microhematocrit capillary tubes filled with CH medicament to confirm resistance to pH change (Foster, et al. 1993). A similar control group was not included in the experiment conducted in extracted teeth, however. No additional studies were identified during literature review where use of a similar negative control was employed. For this investigation, a full wax negative control was added for the purpose of verifying experimental methodology. These 6 samples acted as a secondary negative control to ensure sticky wax provided an adequate seal over the duration of the experimental period (28 days).

One of the six full wax samples (sample #77) demonstrated an unexpected rise in pH relative to similar controls (Figure 8). It appears that this sample was not submerged twice into sticky wax, perhaps contributing to the noted pH rise. The failure of a negative control sample prompted a visual surveillance of all samples after completion of the pH measuring phase. Seven samples were identified as outliers by pH value alone (Table 3). During examination under 8X magnification, 14 of the samples appeared to have a potentially compromised coronal or apical seal based on visual evaluation. The problems were labeled either as a defect in apical seal (AS), coronal seal (CS), or cemental defect (CD) not previously identified (Table 3).

pH outliers were identified visually from each group using line graphs from each group. These data points were all then confirmed to be at least 2 SD's outside the mean

of their respective groups, with the exception of samples 3 and 4 from the UC + group. These two samples were > than 1 SD, but just less than 2 outside the mean of their respective groups. The decision was made to exclude these 6 samples for sensitivity analysis purposes. pH values of outlier and defective seal status could not be correlated. In fact, only three samples were identified (#4, #28, and #77) where outlying pH values correlated with poor wax seal (Table 3). 12 of 15 samples identified as having a compromised wax seal fell within normal distributions in pH values for each respective group. The aim of the sensitivity analysis was to more conservatively identify effects of pH change that might be attributed to the experimental variables.

The negative control is an imperative part of experimental design. Future studies relating to this project may consider running a pilot evaluating sticky wax or a combination of products to verify durability of apical seal over entire planned experimental timeframe. Alternatively, a methodology using microelectrodes to measure liquid placed in wells created on the dentinal root surface may be employed. Several studies have successfully demonstrated pH change using this experimental design (Nerwich, et al., 1993; Chamberlain, et al., 2009). For the current investigation, this was not feasible, as proper electrodes were not available to make necessary measurements.

Statistical Analysis

In order to account for outlying data points and possible failure of the wax seals, a sensitivity analysis was run which removed the six outlying data points. Removing these data points did have a significant impact on findings within the model. The impact was not limited to one area or group of points, but rather shifted areas of significance throughout the model (Tables 4,5,6). The failure of the negative control becomes an undermining feature to the data analysis of this study. The decision was made to evaluate both the original data set as well as the sensitivity data set. The sensitivity data eliminates those samples with the greatest final pH values. By doing this, it was hoped to more conservatively identify the effects of ethanol and medicament on pH change at the external root surface.

Remaining Dentin Thickness (RDT)

The primary experimental question addressed in this study was the effect of a final rinse of 95% ethyl alcohol on diffusion of hydroxyl ions to the external root surface. Because hydroxyl ions are unable to diffuse through intact cementum (Nerwich, et al., 1993), an external defect was made to simulate EIR or mechanical damage after trauma. It has been previously demonstrated that remaining dentin thickness has a significant effect on the permeability of dentin (Pashley, et al., 1988; Fuss, et al., 1989; Nerwich, et al., 1993).

The depth of defects at the external root surface secondary to EIR has not been well characterized in humans. In a study by Laux, 26% of teeth with periapical lesions were diagnosed as having "severe" apical resorption on a histologic level, meaning resorption through cementum and into dentin, but depth was not quantified (Laux, et al., 2000). Several previous studies investigating pH change at the external root surface quantified RDT values as part of the experimental model either before or after pH measurements. Most of the models reported values <2 mm of RDT. Tsesis standardized RDT at three sites along the root surface, with values of 0.4mm, 0.7mm or 1mm established to demonstrate the ability of electrophoretically activated CH to affect faster pH change in a bovine model over traditional CH medicament application (Tsesis, et al., 2005). Nerwich measured an average of 0.97mm inner and 1.66 mm outer apical defects and 1.10mm inner and 2.54mm outer cervical defects (Nerwich, et al., 1993). Javidi used CBCT preoperatively to calculate the required depth of defect in order to standardize 1mm of RDT (Javidi, et al., 2013). Ozdemir used radiographs to measure RDT after a standardized defect of 3mm wide by 1mm deep was cut into the root surface. Average RDT was 1.48±0.13mm, which is also less than the current experimental model (Ozdemir, et al., 2008).

In this study model, defects were created with the intension of removing cementum only. RDT in this study was 2.52 ± 0.39mm with a range of 1.65-3.31mm. This is a substantial range in RDT, and represents a significantly higher mean RDT value compared to the studies previously described. Outer layers of dentin are less permeable which may inhibit complete diffusion of available hydroxyl ions. pH change in this

model, therefore, may be more meaningful from a clinical perspective as the depth of dentin defects is typically unknown during treatment as well.

RDT was considered as part of the statistical model to assess its effect on pH change. As seen in Fig 13, there was a statistically significant correlation between pH and dentinal thickness found in this study. Increased dentin thickness correlated to lower pH's measured at the root surface via the saline storage solution. This is in agreement with Pashley et al., (1988), Outhwaite et al., (1976), and Nerwich et al., (1993), all of whom discuss how increase dentin thickness is a major factor affecting dentin permeability.

Tubule density and diameter increases progressing from outer dentin to inner dentin. (Thomas1985). Cervical root dentin is approximately 10 times less permeable than coronal dentin (Pashley, et al., 1988). There is a continuous decrease in tubule density from coronal to apical extent of the root canal. Diffusion rate increases exponentially at the inner layers of dentin, whereas dentinal thickness has a linear relationship to permeability at the outer most layers of dentin (Outhwaite, et al., 1976).

Because of the great variability in the permeability of dentin depending on depth and location of the defect created, controlling for RDT may have more accurately captured the effects of ethanol as a final irrigant on pH change at the external root surface. Although shallow defects may be clinically significant, it is important to limit the influence of this independent variable in order to more accurately measure the effect of the variable of choice, in this case ethanol's affect on pH. This should be a consideration for future study design.

pH Drop

Previous studies have concluded that CH will need to be replenished on a monthly interval in order to maintain clinically relevant pH levels (Chamberlain et al., 2009). Others have proposed that CH medicaments are capable of maintaining high pH levels for extended time periods up to 120 days (Esberard, et al. 1996). In this investigation there was a consistent drop of pH starting at day 12 in all treatment groups (Figures 9-12). The drop in pH was noted in all groups, including negative controls that were not

treated with any medicament. There are several plausible explanations of this drop in pH noted in the experimental model.

pH change occurs as a result of dissociation of CH into Ca^{2+} and OH^- ions. The hydroxyl ions then diffuse through dentinal tubules to the saline at the external root surface causing pH change. CH was not replenished at any time point during the investigation. A plausible explanation for pH drop at day 12 is that maximum dissociation of medicament had occurred and therefore minimal diffusion could take place after day 12. A similar finding was noted in the BC sealer groups. The same mechanism could account for this drop, indicating the CH produced during the hydration reaction was depleted at by day 12.

A similar drop was observed in the negative control groups without placement of medicament. The negative control group provides a baseline to compare with active treatment groups. There appears to be some interaction between dentin and the saline storage medium causing a slightly negative trend in pH over the course of the experiment (Figure 12a). Similar trends were noted in previous investigations where all samples returned to baseline levels or below (Fuss, et al., 1989; Calt, et al., 1999). This may explain why treatment groups did not reach a plateau at maximum pH values in a closed system, but rather started a return towards baseline of the negative control groups after maximal dissociation of medicament had occurred.

There is a clinically significant implication of this pH drop in the CH treatment groups. The maximum pH values were reached by day 12 in all treatment groups, after which minimal diffusion of hydroxyl ions could be observed via pH change in the saline storage solution. If maximum pH is to be reached and maintained in treatment of existing EIR, CH medicament may need to be replenished between 2-3 weeks after application. This may make the initial surge in pH of the ethanol positive groups even more relevant clinically as an alkaline environment could be more quickly established using ethanol as a final rinse.

CONCLUSIONS

It was possible to demonstrate diffusion of hydroxyl ions to the external root surface after smear layer removal and final rinse with ethyl alcohol in an extracted tooth model. Data trends reported in this study demonstrate an increase in rate of change of pH with the use of 95% ethyl alcohol as a final rinse prior to CH medicament application. The use of 95% ethyl alcohol does not appear to increase the magnitude of pH change after medicament application. BC sealer may maintain alkalinity for a longer duration when 95% ethyl alcohol is used as a final rinse. Finally, pH values appear to decrease after 12 days in this *in vitro* model, indicating a potential need to replenish CH after this timeframe in longer term treatment cases.

Remaining dentin thickness was confirmed as a significant independent variable that must be considered in future study design. Future experiments looking to isolate the effect of 95% ethyl alcohol on pH change at the external root structure should consider changes in methodology to control for RDT. Consider selecting teeth that are from patients of similar age ranges and tooth types if possible. Finally, an effort should be made to confirm if an adequate seal could be created by application of sticky wax when submerging samples in saline for approximately 1 month.

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APPENDIX 1 - pH all samples

pH – Raw Data collected at Each Time Point Data Listed for All 10 Groups												
Group	Sample	**0**	**1**	**2**	**4**	**7**	**9**	**12**	**16**	**19**	**22**	**28**
UC (+)	pH 7 Buffer			7.03	7.03	7.03	7.03	7.01	7.01	6.99	7.03	7.02
	1	6.68	7.05	6.80	7.14	7.05	6.93	6.61	6.74	6.74	6.86	6.87
	2	6.57	7.06	7.08	7.21	7.13	7.17	7.13	7.23	7.13	7.16	7.13
	3	6.70	7.26	7.26	7.20	7.33	7.59	8.39	8.03	8.50	8.42	8.04
Group	4	6.78	7.21	7.27	7.20	7.75	7.85	8.07	8.09	8.53	8.50	8.22
#1	5	6.82	8.22	8.29	8.17	8.55	8.82	7.35	6.94	6.90	6.92	6.77
	6	7.24	7.42	7.29	7.14	7.20	7.18	6.98	6.81	6.78	6.79	6.69
	7	6.81	7.23	7.14	7.06	6.85	6.82	6.65	6.67	6.71	6.79	6.91
	8	8.82	7.47	7.29	7.16	7.16	6.98	6.83	6.84	6.88	6.98	6.92
	9	7.41	8.11	7.93	7.77	7.64	7.03	6.78	6.63	6.62	6.71	6.67
	10	7.62	7.81	7.64	8.17	8.77	8.66	7.16	6.85	6.72	6.76	6.71
	Mean	7.15	7.48	7.40	7.42	7.54	7.50	7.20	7.08	7.15	7.19	7.09
	SD	0.68	0.42	0.44	0.44	0.65	0.72	0.60	0.54	0.73	0.68	0.57
UC(-)	pH 7 Buffer			7.01	7.01	7.02	7.01	7.00	7.02	7.01	7.03	7.03
	11	7.59	7.32	7.18	7.15	7.23	7.39	6.90	6.84	6.92	7.17	7.14
	12	7.46	7.32	7.20	7.12	7.30	7.23	7.08	6.98	7.00	7.12	7.29
	13	7.07	7.13	7.12	7.85	8.67	8.84	7.99	7.17	7.15	7.20	7.22
Group	14	7.30	7.24	7.24	7.18	7.38	7.63	7.10	7.06	7.11	7.18	7.23
#2	15	7.46	7.51	7.54	8.28	8.95	9.32	9.70	10.26	10.52	10.58	10.78
	16	7.12	7.39	7.29	7.12	7.12	7.13	6.94	6.92	7.01	7.10	7.08
	17	7.32	7.37	7.20	7.10	7.24	7.21	7.05	6.98	7.04	7.17	7.16
	18	7.38	7.44	7.41	7.12	7.16	7.18	6.92	6.88	6.96	7.11	7.08
	19	7.55	7.35	7.23	7.09	7.10	7.30	7.16	7.07	7.11	7.17	7.18
	20	7.58	7.45	7.17	7.27	8.21	8.84	8.11	7.27	7.25	7.29	7.25
	Mean	7.38	7.35	7.26	7.33	7.64	7.81	7.50	7.34	7.41	7.51	7.54
	SD	0.18	0.11	0.13	0.40	0.70	0.85	0.89	1.03	1.10	1.08	1.14
CS (+)	pH 7 Buffer			7.03	7.02	7.02	7.02	7.02	7.02	7.02	7.03	7.01
	21	7.15	7.57	7.36	7.62	7.71	7.31	8.83	7.50	7.00	7.03	6.95
	22	7.64	7.86	7.63	7.17	7.10	7.09	7.28	6.87	6.74	6.79	6.64
	23	6.78	7.87	7.69	7.57	7.61	7.69	8.13	7.06	6.82	6.84	6.72
Group	24	7.12	7.63	7.50	7.37	7.35	7.62	7.81	6.46	6.19	6.07	6.08
#3	25	6.84	7.68	7.34	7.13	7.09	7.16	7.25	6.84	6.61	6.62	6.50
	26	7.98	8.49	8.20	8.01	7.95	7.89	7.66	7.53	6.87	6.85	6.66

	27	6.94	7.58	7.42	8.04	8.70	9.13	8.50	7.95	7.01	7.60	6.86
	28	6.93	8.71	9.12	9.45	9.56	9.77	10.11	10.35	10.52	10.52	10.69
	29	7.17	8.30	8.28	8.59	8.73	8.87	9.16	8.14	7.08	7.13	6.98
	30	7.27	7.59	7.35	7.13	7.10	7.15	7.42	6.90	6.80	6.86	6.81
	Mean	7.18	7.93	7.79	7.81	7.89	7.97	8.22	7.56	7.16	7.23	7.09
	SD	0.37	0.42	0.58	0.75	0.85	0.95	0.93	1.11	1.21	1.22	1.29
CS(-)	pH 7 Buffer			7.02	7.03	7.01	7.01	7.02	7.04	7.04	7.02	7.02
	31	6.99	7.43	7.33	7.46	7.23	7.33	7.91	7.47	6.94	6.92	6.94
	32	9.16	7.59	7.34	7.13	7.04	7.04	7.07	6.93	6.87	7.03	6.98
	33	7.61	7.69	7.51	7.43	7.73	8.19	8.72	8.95	8.32	8.03	7.69
Group	34	7.06	7.63	7.55	7.12	7.38	7.52	7.46	8.10	8.29	8.09	7.78
#4	35	7.16	7.47	7.30	7.10	7.19	7.30	8.02	7.26	7.24	7.26	7.09
	36	7.32	7.54	7.45	7.21	7.18	7.34	7.49	7.04	7.08	7.17	7.12
	37	7.82	7.50	7.43	7.37	7.55	8.06	8.28	8.10	7.29	7.16	7.24
	38	7.80	7.47	7.44	7.41	7.74	7.92	8.52	7.43	7.70	7.22	6.91
	39	7.59	7.45	7.23	7.29	8.02	8.52	8.57	7.48	7.34	7.41	7.22
	40	7.69	7.59	7.37	7.33	7.68	8.15	8.74	8.94	9.28	9.59	10.08
	Mean	7.62	7.54	7.40	7.29	7.47	7.74	8.08	7.77	7.64	7.59	7.51
	SD	0.62	0.09	0.10	0.14	0.32	0.49	0.59	0.73	0.77	0.81	0.95
BC (+)	pH 7 Buffer			7.01	7.02	7.02	7.03	7.01	7.03	6.97	7.03	7.01
	41	8.05	7.83	7.27	7.30	7.27	7.26	7.32	6.46	6.29	6.25	6.23
	42	9.34	8.89	8.41	8.03	7.88	7.90	7.89	6.59	6.44	6.37	6.22
	43	8.15	8.06	7.75	7.67	7.48	7.47	7.74	6.56	6.49	6.40	6.22
Group	44	8.07	7.86	7.79	7.55	7.41	7.38	7.42	6.50	6.25	6.14	6.09
#5	45	7.98	7.72	7.62	7.31	7.21	7.29	7.21	6.43	6.23	6.21	6.15
	46	8.41	7.85	7.58	7.46	7.29	7.37	7.34	6.44	6.43	6.27	6.22
	47	8.34	7.77	7.48	8.03	8.91	9.19	9.66	10.07	10.08	10.18	10.40
	48	7.92	7.88	7.71	7.77	7.70	7.76	7.72	6.60	6.48	6.42	6.44
	49	8.78	8.29	7.96	7.85	7.63	7.60	7.60	6.57	6.68	6.72	6.34
	50	8.15	7.71	7.38	7.25	7.31	7.32	7.41	6.71	6.33	6.58	6.51
	Mean	8.32	7.99	7.70	7.62	7.61	7.65	7.73	6.89	6.77	6.75	6.68
	SD	0.44	0.36	0.32	0.29	0.51	0.58	0.71	1.12	1.17	1.22	1.31
BC (-)	pH 7 Buffer			7.01	7.02	7.02	7.02	7.01	7.02	6.97	7.02	7.00
	51	8.18	7.72	7.56	7.15	7.32	7.25	7.29	6.53	5.98	5.91	5.75
	52	7.80	7.80	7.74	7.49	7.48	7.35	7.35	6.59	6.08	6.02	5.85
	53	8.69	7.82	7.64	7.45	7.31	7.27	7.19	6.52	6.00	5.96	5.77

Group	54	7.96	7.60	7.49	7.49	7.35	7.35	7.40	6.88	6.20	6.07	5.89
#6	55	7.71	7.67	7.46	7.40	7.34	7.37	7.40	6.53	6.01	5.87	5.77
	56	7.81	7.70	7.52	7.42	7.35	7.34	7.42	6.57	6.01	5.91	5.80
	57	7.88	7.68	7.54	7.49	7.39	7.29	7.32	6.72	6.13	6.04	5.89
	58	8.63	8.01	7.62	7.47	7.40	7.45	7.40	6.91	6.09	5.97	5.83
	59	8.28	8.10	7.98	7.79	7.79	7.82	8.04	7.31	6.45	6.03	5.85
	60	8.30	8.04	7.74	7.58	7.49	7.46	7.48	6.65	6.07	6.00	5.79
	Mean	8.12	7.81	7.63	7.47	7.42	7.40	7.43	6.72	6.10	5.98	5.82
	SD	0.35	0.18	0.16	0.16	0.14	0.16	0.23	0.25	0.14	0.07	0.05
NC (+)	pH 7 Buffer			6.98	7.01	7.01	7.02	7.01	7.02	7.00	7.03	7.00
	61	7.50	7.50	7.43	7.19	7.12	6.71	6.32	6.22	6.15	6.23	6.18
	62	7.64	7.58	7.49	7.38	7.30	7.29	6.34	6.21	6.17	6.24	6.17
	63	7.68	7.49	7.49	7.42	7.29	7.32	6.23	6.12	6.21	6.41	6.42
Group	64	7.64	7.50	7.53	7.45	7.54	7.42	6.31	6.13	6.14	6.14	6.49
#7	65	7.77	7.60	7.50	7.36	7.28	7.27	6.38	6.15	6.13	6.25	6.38
	66	7.57	7.57	7.42	7.44	7.46	7.30	6.13	6.13	6.14	6.18	6.73
	SD	0.09	0.05	0.04	0.10	0.15	0.25	0.09	0.04	0.03	0.09	0.21
FW	67	7.73	7.54	7.38	7.04	7.09	7.33	6.60	6.47	6.39	6.47	6.28
FW	68	7.01	6.86	6.82	6.66	6.99	6.91	6.75	6.74	6.65	6.94	6.30
FW	69	7.14	7.12	7.03	7.04	7.26	7.13	6.62	6.46	6.38	6.60	6.42
	Mean	7.29	7.17	7.08	6.91	7.11	7.12	6.66	6.56	6.47	6.67	6.33
	SD	0.38	0.34	0.28	0.22	0.14	0.21	0.08	0.16	0.15	0.24	0.08
NC (-)	pH 7 Buffer			7.02	7.01	7.01	7.02	7.01	7.02	6.97	7.02	7.02
	71	7.75	7.42	7.37	7.20	7.17	7.18	7.14	6.78	6.39	6.45	6.34
	72	7.79	7.65	7.50	7.34	7.28	6.94	6.69	6.61	6.50	6.57	6.46
	73	7.77	7.67	7.57	7.42	7.33	7.29	7.20	6.81	6.32	6.27	6.22
Group	74	7.76	7.67	7.47	7.40	7.29	7.20	6.86	6.65	6.47	6.28	6.04
#8	75	7.75	7.58	7.54	7.42	7.28	7.27	6.85	6.71	6.46	6.48	6.32
	76	7.57	7.53	7.50	7.32	7.14	7.08	7.13	6.53	6.34	6.39	6.45
	SD	0.08	0.10	0.07	0.08	0.08	0.13	0.21	0.11	0.07	0.12	0.16
FW	77	7.65	7.52	7.35	8.06	9.64	10.12	10.81	11.09	11.14	11.13	11.22
FW	78	7.08	7.27	7.19	7.42	7.52	7.39	7.09	6.83	7.00	7.22	7.03
FW	79	8.11	7.61	7.47	7.56	7.49	7.46	5.71	5.40	5.35	5.30	5.24
	Mean	7.61	7.47	7.34	7.68	8.22	8.32	7.87	7.77	7.83	7.88	7.83
	SD	0.52	0.18	0.14	0.34	1.23	1.56	2.64	2.96	2.98	2.97	3.07
Saline	pH 7 Buffer			7.02	7.01	7.01	7.01	7.01	7.01	6.98	7.02	7.01

	81	7.07	7.05	7.04	6.98	7.26	7.05	7.12	7.22	6.98	6.87	6.84
	82	7.24	7.22	7.02	7.26	7.37	7.15	7.30	7.17	7.06	7.20	7.05
	83	7.19	7.11	7.16	7.24	7.31	7.35	7.22	7.12	7.04	7.22	7.13
Group	84	7.00	6.99	7.09	7.22	7.20	7.18	7.09	7.05	6.96	7.07	7.02
#9	85	7.10	7.05	6.99	7.24	7.31	7.28	7.27	7.12	7.02	7.22	7.14
	80 (8)	7.67	7.47	7.01	7.31	7.36	7.44	7.01	6.94	6.99	7.04	6.74
	70 (7)	7.09	7.08	6.92	7.26	7.20	7.26	7.06	7.05	7.01	7.09	7.07
	Mean	7.19	7.14	7.03	7.22	7.29	7.24	7.15	7.10	7.01	7.10	7.00
	SD	0.22	0.16	0.08	0.11	0.07	0.13	0.11	0.09	0.03	0.13	0.15
FULL WAX												
	67 (UC+)	7.73	7.54	7.38	7.04	7.09	7.33	6.60	6.47	6.39	6.47	6.28
	68 (CS+)	7.01	6.86	6.82	6.66	6.99	6.91	6.75	6.74	6.65	6.94	6.30
Group	69 (BC+)	7.14	7.12	7.03	7.04	7.26	7.13	6.62	6.46	6.38	6.60	6.42
#10	77 (UC-)	7.65	7.52	7.35	8.06	9.64	10.12	10.81	11.09	11.14	11.13	11.22
	78 (CS-)	7.08	7.27	7.19	7.42	7.52	7.39	7.09	6.83	7.00	7.22	7.03
	79 (BC-)	8.11	7.61	7.47	7.56	7.49	7.46	5.71	5.40	5.35	5.30	5.24

APPENDIX II – Average Dentin Thickness

(CS=coronal seal, AS=apical seal, CD=cementum damage)

AVERAGE DENTIN THICKNESS (mm) 4 MEASUREMENTS MADE AT EACH DEFECT SITE						
Samples	**Descriptors**	**Coronal 1**	**Coronal 2**	**Apical 2**	**Apical 2**	**Mean**
1	none	2.01	2.20	2.31	3.17	2.42
2	AS	2.65	3.35	2.31	3.17	2.87
3	none	2.20	2.70	2.30	2.09	2.32
4	CS	1.49	1.65	1.59	2.10	1.71
5	none	2.26	2.44	1.69	1.97	2.09
6	none	2.87	2.24	2.24	2.65	2.50
7	none	2.91	3.22	2.65	3.38	3.04
8	CS	2.33	2.41	1.79	1.87	2.10
9	none	2.95	2.84	2.36	2.54	2.67
10	none	3.34	2.75	3.00	2.52	2.90
11	none	2.42	2.58	2.26	2.92	2.55
12	none	2.53	2.99	2.42	2.78	2.68
13	none	1.64	2.01	1.61	2.06	1.83
14	none	3.01	2.52	2.59	2.78	2.73
15	CD	1.98	2.08	1.94	2.01	2.00
16	AS	2.31	2.67	2.01	2.53	2.38
17	none	1.94	2.38	2.40	1.93	2.16
18	AS	2.70	2.52	2.28	2.40	2.48
19	none	2.70	2.13	2.36	1.89	2.27
20	none	2.12	2.41	2.57	2.00	2.28
21	none	2.16	2.20	1.69	1.87	1.98
22	none	2.56	2.94	2.42	2.09	2.50
23	none	3.00	2.27	2.45	2.56	2.57
24	none	2.41	2.30	2.27	2.66	2.41
25	none	2.76	2.77	2.92	2.44	2.72
26	AS	2.49	2.24	2.26	2.32	2.33
27	none	2.30	2.23	2.38	2.23	2.29
28	AS	2.32	2.17	2.03	1.99	2.13
29	none	2.47	2.72	2.11	2.48	2.45
30	none	2.28	2.90	2.67	2.53	2.60
31	none	2.77	2.42	2.21	2.22	2.41
32	CS	2.54	2.56	2.14	2.49	2.43
33	none	2.65	2.86	2.32	2.64	2.62

34	none	2.89	2.96	2.32	2.64	2.70
35	none	3.40	3.15	2.78	2.87	3.05
36	AS	3.68	2.73	2.37	3.23	3.00
37	none	2.38	2.71	2.25	2.91	2.56
38	CS	3.34	3.61	2.54	3.17	3.17
39	none	2.11	2.56	2.20	2.43	2.75
40	none	2.73	2.99	2.44	2.62	2.70
41	none	2.63	3.03	2.72	3.19	2.89
42	none	2.22	1.96	1.49	1.71	1.85
43	none	2.54	2.75	2.57	2.68	2.64
44	none	3.40	3.17	3.05	3.33	3.24
45	none	2.65	2.78	2.11	2.87	2.60
46	none	2.60	2.92	2.45	2.35	2.58
47	none	1.48	1.82	1.47	1.82	1.65
48	none	2.50	3.11	2.15	2.24	2.50
49	none	2.02	2.69	1.65	2.36	2.18
50	none	3.05	2.08	2.21	2.80	2.36
51	none	2.64	2.69	2.22	2.87	2.61
52	none	2.09	1.89	1.62	1.68	1.82
53	none	3.68	3.28	2.87	3.41	3.31
54	none	1.97	2.47	1.86	3.00	2.33
55	none	2.16	3.31	2.74	3.12	2.83
56	none	3.10	3.50	3.03	3.08	3.18
57	none	2.51	3.10	2.31	2.69	2.92
58	none	1.86	1.86	1.75	1.91	1.85
59	none	1.73	2.01	1.77	1.72	1.81
60	none	2.48	2.77	2.37	2.54	2.54
61	none	2.54	2.88	2.96	2.58	2.74
62	none	2.90	3.51	2.89	2.54	2.96
63	none	3.27	2.85	2.15	2.77	2.76
64	none	3.52	3.94	2.00	3.44	3.23
65	none	2.94	2.58	2.66	2.17	2.59
66	none	2.44	2.73	2.79	2.48	2.61
67	AS	2.09	2.63	1.91	2.55	2.30
68	none	2.63	2.54	2.59	2.67	2.61
69	none	3.74	2.82	2.56	2.61	2.93
70(saline)	-	-	-	-	-	

71	none	3.01	3.36	2.69	2.22	2.82
72	none	2.11	2.37	1.72	1.95	2.04
73	none	2.64	3.19	2.48	3.39	2.93
74	none	2.71	2.79	2.94	3.25	2.92
75	none	3.20	2.65	2.80	2.75	2.85
76	none	2.29	2.00	1.75	1.88	1.98
77	CS/AS	3.34	2.51	2.63	2.42	2.73
78	none	1.81	1.74	1.71	1.63	1.72
79	none	2.82	2.81	2.25	2.37	2.56
80 (saline)	-	-	-	-	-	

APPENDIX III – Multiple Linear Regression Data

Summary of Means and SE Multiple Linear Regression – Original and Sensitivity Analysis						
Time (days)	**Medicament**	**Etoh**	**Mean**	**SE original**	**Mean sensitivity analysis**	**SE sensitivity analysis**
0	BC	+	8.31	0.14	8.31	0.10
0	BC	-	8.12	0.14	8.12	0.10
0	CS	+	7.16	0.14	7.10	0.11
0	CS	-	7.66	0.14	7.43	0.12
0	NC	+	7.69	0.18	7.66	0.13
0	NC	-	7.75	0.18	7.74	0.13
0	UC	+	7.13	0.14	7.04	0.12
0	UC	-	7.35	0.14	7.39	0.12
1	BC	+	7.98	0.09	7.97	0.08
1	BC	-	7.81	0.09	7.81	0.08
1	CS	+	7.91	0.09	7.75	0.09
1	CS	-	7.57	0.09	7.56	0.09
1	NC	+	7.58	0.11	7.58	0.10
1	NC	-	7.60	0.11	7.59	0.10
1	UC	+	7.48	0.09	7.59	0.09
1	UC	-	7.32	0.09	7.29	0.09
2	BC	+	7.68	0.10	7.68	0.08
2	BC	-	7.63	0.10	7.63	0.08
2	CS	+	7.77	0.10	7.56	0.09
2	CS	-	7.43	0.10	7.41	0.10
2	NC	+	7.52	0.13	7.51	0.11
2	NC	-	7.50	0.13	7.50	0.10
2	UC	+	7.39	0.10	7.48	0.10
2	UC	-	7.23	0.10	7.17	0.10
4	BC	+	7.60	0.12	7.60	0.09
4	BC	-	7.47	0.12	7.47	0.09
4	CS	+	7.77	0.12	7.55	0.10
4	CS	-	7.34	0.12	7.34	0.11
4	NC	+	7.45	0.16	7.44	0.12
4	NC	-	7.37	0.15	7.36	0.12
4	UC	+	7.41	0.12	7.53	0.11
4	UC	-	7.28	0.12	7.20	0.11
7	BC	+	7.58	0.16	7.57	0.14
7	BC	-	7.42	0.16	7.41	0.14
7	CS	+	7.83	0.16	7.63	0.16

Summary of Means and SE Multiple Linear Regression – Original and Sensitivity Analysis						
Time (days)	**Medicament**	**Etoh**	**Mean**	**SE original**	**Mean sensitivity analysis**	**SE sensitivity analysis**
7	CS	-	7.58	0.16	7.60	0.17
7	NC	+	7.47	0.21	7.45	0.19
7	NC	-	7.28	0.21	7.27	0.18
7	UC	+	7.52	0.16	7.64	0.17
7	UC	-	7.55	0.16	7.51	0.17
9	BC	+	7.62	0.19	7.61	0.17
9	BC	-	7.39	0.19	7.39	0.17
9	CS	+	7.91	0.19	7.71	0.19
9	CS	-	7.85	0.19	7.93	0.21
9	NC	+	7.37	0.25	7.35	0.23
9	NC	-	7.20	0.25	7.18	0.22
9	UC	+	7.47	0.19	7.59	0.21
9	UC	-	7.71	0.19	7.69	0.21
12	BC	+	7.68	0.19	7.68	0.15
12	BC	-	7.43	0.19	7.42	0.15
12	CS	+	8.13	0.19	7.99	0.17
12	CS	-	8.22	0.19	8.33	0.18
12	NC	+	6.48	0.25	6.44	0.20
12	NC	-	7.02	0.24	7.01	0.19
12	UC	+	7.16	0.19	7.15	0.18
12	UC	-	7.37	0.19	7.24	0.18
16	BC	+	6.84	0.23	6.84	0.18
16	BC	-	6.72	0.23	6.71	0.18
16	CS	+	7.46	0.23	7.16	0.20
16	CS	-	7.95	0.24	8.13	0.21
16	NC	+	6.40	0.31	6.33	0.23
16	NC	-	6.74	0.30	6.71	0.23
16	UC	+	7.04	0.23	6.97	0.21
16	UC	-	7.20	0.24	6.94	0.21
19	BC	+	6.71	0.25	6.72	0.19
19	BC	-	6.10	0.25	6.09	0.18
19	CS	+	7.07	0.26	6.73	0.21
19	CS	-	7.81	0.26	7.89	0.22
19	NC	+	6.39	0.34	6.31	0.25
19	NC	-	6.47	0.33	6.44	0.24
19	UC	+	7.10	0.25	7.01	0.22
19	UC	-	7.26	0.26	6.98	0.22
22	BC	+	6.70	0.26	6.70	0.20

Summary of Means and SE Multiple Linear Regression – Original and Sensitivity Analysis						
Time (days)	**Medicament**	**Etoh**	**Mean**	**SE original**	**Mean sensitivity analysis**	**SE sensitivity analysis**
22	BC	-	5.98	0.26	5.97	0.19
22	CS	+	7.13	0.26	6.81	0.22
22	CS	-	7.77	0.26	7.86	0.23
22	NC	+	6.48	0.34	6.39	0.26
22	NC	-	6.46	0.33	6.43	0.25
22	UC	+	7.14	0.26	7.05	0.23
22	UC	-	7.36	0.26	7.09	0.24
28	BC	+	6.62	0.27	6.63	0.21
28	BC	-	5.82	0.27	5.81	0.21
28	CS	+	6.99	0.28	6.64	0.23
28	CS	-	7.68	0.28	7.80	0.25
28	NC	+	6.63	0.36	6.54	0.28
28	NC	-	6.36	0.35	6.33	0.27
28	UC	+	7.05	0.27	6.96	0.25
28	UC	-	7.39	0.28	7.11	0.25

APPENDIX IV - Multiple Linear Regression Model - Comparing Effects of Ethanol, Medicament, Interaction, and RDT

LEGEND FOR TABLE

(medicament) - Main Effect of Medicament
(etoh) - Main Effect of EtOH
(medicament*etoh) - Interaction – (If significant, then differences between Medicaments should be described separately for the +/- EtOH groups)
(avgdis) - Association between RDT and pH

Fields highlighted in RED = p value <0.05 in original
Fields highlighted in YELLOW = p value < 0.05 in sensitivity analysis

Multiple Linear Regression Effects Model – EtOH, Medicament, Interaction, RDT			
TIME	**SOURCE**	**P-VALUE**	**P-VALUE SENSITIVITY ANALYSIS**
0	medicament	**0.0000**	**0.0000**
0	etoh	0.1617	0.0896
0	medicament*etoh	0.1041	**0.0490**
0	avgdis	0.1681	0.3857
1	medicament	**0.0000**	**0.0000**
1	etoh	**0.0178**	**0.0166**
1	medicament*etoh	0.3836	0.4464
1	avgdis	0.0938	0.1076
2	medicament	**0.0043**	**0.0058**
2	etoh	0.0562	**0.0490**
2	medicament*etoh	0.4296	0.4191
2	avgdis	0.1199	0.1441
4	medicament	0.2285	0.3901
4	etoh	**0.0359**	**0.0178**
4	medicament*etoh	0.5051	0.7152
4	avgdis	**0.0271**	**0.0182**
7	medicament	0.3273	0.4918
7	etoh	0.2519	0.3019
7	medicament*etoh	0.8335	0.9734
7	avgdis	**0.0063**	**0.0108**
9	medicament	0.0531	0.0714
9	etoh	0.7142	0.9239
9	medicament*etoh	0.6401	0.6012
9	avgdis	**0.0106**	**0.0204**
12	medicament	**0.0000**	**0.0000**

Multiple Linear Regression Effects Model – EtOH, Medicament, Interaction, RDT			
12	etoh	0.3065	0.1426
12	medicament*etoh	0.3227	0.0942
12	avgdis	**0.0012**	**0.0012**
16	medicament	**0.0002**	**0.0000**
16	etoh	0.2256	0.0413
16	medicament*etoh	0.6228	0.0307
16	avgdis	**0.0013**	**0.0036**
19	medicament	**0.0002**	**0.0000**
19	etoh	0.6408	0.2939
19	medicament*etoh	0.0814	**0.0006**
19	avgdis	**0.0034**	**0.0110**
22	medicament	**0.0001**	**0.0000**
22	etoh	0.8826	0.5478
22	medicament*etoh	0.0729	**0.0014**
22	avgdis	**0.0028**	**0.0142**
28	medicament	**0.0002**	**0.0001**
28	etoh	0.9627	0.6959
28	medicament*etoh	**0.0451**	**0.0008**
28	avgdis	**0.0052**	**0.0253**

Appendix V – Multiple Linear Regression – Additional comparisons

LEGEND FOR TABLE

At each time point (0-28) the following comparisons were made

1. Comparing BC+Etoh vs BC-Etoh
2. Comparing CS+Etoh vs CS-Etoh
3. Comparing NC+Etoh vs NC-Etoh
4. Comparing UC+Etoh vs UC-Etoh
5. Comparing BC/CS/UC/NC/ - All received EtOH
6. Comparing BC/CS/UC/NC/ - All did NOT receive EtOH

Fields highlighted in RED = p value <0.05 in original
Fields highlighted in YELLOW = p value < 0.05 in sensitivity analysis

time	medicament	etoh	P-value	P-value from sensitivity analysis
0	BC		0.3529	0.1861
0	CS		0.0137	0.0519
0	NC		0.8297	0.6772
0	UC		0.2758	0.0434
0		+	0.0000	0.0000
0		-	0.0023	0.0000
1	BC		0.1904	0.1504
1	CS		0.0085	0.1542
1	NC		0.9356	0.9211
1	UC		0.2215	0.0270
1		+	0.0002	0.0072
1		-	0.0025	0.0011
2	BC		0.6910	0.6229
2	CS		0.0189	0.2675
2	NC		0.9097	0.9192
2	UC		0.2475	0.0262
2		+	0.0401	0.3948
2		-	0.0430	0.0067
4	BC		0.4404	0.3239
4	CS		0.0151	0.1688
4	NC		0.6967	0.6403
4	UC		0.4441	0.0464
4		+	0.1517	0.7981
4		-	0.7033	0.3536
7	BC		0.4988	0.4398
7	CS		0.2726	0.8973

time	medicament	etoh	P-value	P-value from sensitivity analysis
7	NC		0.5259	0.5000
7	UC		0.8795	0.6120
7		+	0.4404	0.8720
7		-	0.6581	0.5863
9	BC		0.4100	0.3564
9	CS		0.8390	0.4290
9	NC		0.6205	0.6111
9	UC		0.3771	0.7171
9		+	0.2959	0.6865
9		-	0.1274	0.0663
12	BC		0.3434	0.2203
12	CS		0.7501	0.1804
12	NC		0.1217	0.0445
12	UC		0.4215	0.7175
12		+	0.0000	0.0000
12		-	0.0012	0.0000
16	BC		0.7276	0.6035
16	CS		0.1581	0.0015
16	NC		0.4277	0.2355
16	UC		0.6315	0.9389
16		+	0.0532	0.0641
16		-	0.0020	0.0000
19	BC		0.0941	0.0197
19	CS		0.0494	0.0004
19	NC		0.8680	0.6957
19	UC		0.6671	0.9366
19		+	0.2959	0.2205
19		-	0.0001	0.0000
22	BC		0.0513	0.0099
22	CS		0.0929	0.0020
22	NC		0.9692	0.9099
22	UC		0.5509	0.9131
22		+	0.2923	0.3049
22		-	0.0000	0.0000
28	BC		0.0421	0.0074
28	CS		0.0872	0.0015
28	NC		0.5899	0.5853
28	UC		0.3783	0.6803
28		+	0.6213	0.6572
28		-	0.0000	0.0000

APPENDIX VI – pairwise comparisons between each group original data (p-values listed)

time	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
0	**BC+**		0.35	0.00	0.00	0.01	0.02	0.00	0.00
0	**BC-**	0.35		0.00	0.02	0.06	0.09	0.00	0.00
0	**CS+**	0.00	0.00		0.01	0.02	0.01	0.90	0.33
0	**CS-**	0.00	0.02	0.01		0.90	0.71	0.01	0.12
0	**NC+**	0.01	0.06	0.02	0.90		0.83	0.02	0.14
0	**NC-**	0.02	0.09	0.01	0.71	0.83		0.01	0.08
0	**UC+**	0.00	0.00	0.90	0.01	0.02	0.01		0.28
0	**UC-**	0.00	0.00	0.33	0.12	0.14	0.08	0.28	
1	**BC+**		0.19	0.59	0.00	0.01	0.01	0.00	0.00
1	**BC-**	0.19		0.44	0.05	0.11	0.13	0.01	0.00
1	**CS+**	0.59	0.44		0.01	0.03	0.03	0.00	0.00
1	**CS-**	0.00	0.05	0.01		0.91	0.84	0.45	0.06
1	**NC+**	0.01	0.11	0.03	0.91		0.94	0.45	0.08
1	**NC-**	0.01	0.13	0.03	0.84	0.94		0.39	0.06
1	**UC+**	0.00	0.01	0.00	0.45	0.45	0.39		0.22
1	**UC-**	0.00	0.00	0.00	0.06	0.08	0.06	0.22	
2	**BC+**		0.69	0.53	0.07	0.33	0.26	0.04	0.00
2	**BC-**	0.69		0.31	0.16	0.51	0.43	0.09	0.01
2	**CS+**	0.53	0.31		0.02	0.14	0.10	0.01	0.00
2	**CS-**	0.07	0.16	0.02		0.56	0.65	0.78	0.17
2	**NC+**	0.33	0.51	0.14	0.56		0.91	0.42	0.08
2	**NC-**	0.26	0.43	0.10	0.65	0.91		0.48	0.09
2	**UC+**	0.04	0.09	0.01	0.78	0.42	0.48		0.25
2	**UC-**	0.00	0.01	0.00	0.17	0.08	0.09	0.25	
4	**BC+**		0.44	0.31	0.14	0.45	0.23	0.24	0.06
4	**BC-**	0.44		0.08	0.45	0.92	0.59	0.69	0.25
4	**CS+**	0.31	0.08		0.02	0.11	0.04	0.03	0.00
4	**CS-**	0.14	0.45	0.02		0.57	0.90	0.72	0.70
4	**NC+**	0.45	0.92	0.11	0.57		0.70	0.81	0.38
4	**NC-**	0.23	0.59	0.04	0.90	0.70		0.85	0.64
4	**UC+**	0.24	0.69	0.03	0.72	0.81	0.85		0.44
4	**UC-**	0.06	0.25	0.00	0.70	0.38	0.64	0.44	
7	**BC+**		0.50	0.26	1.00	0.69	0.26	0.79	0.91
7	**BC-**	0.50		0.07	0.50	0.86	0.59	0.68	0.57
7	**CS+**	0.26	0.07		0.27	0.18	0.04	0.17	0.22
7	**CS-**	1.00	0.50	0.27		0.68	0.27	0.80	0.92

time	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
7	**NC+**	0.69	0.86	0.18	0.68		0.53	0.86	0.76
7	**NC-**	0.26	0.59	0.04	0.27	0.53		0.37	0.31
7	**UC+**	0.79	0.68	0.17	0.80	0.86	0.37		0.88
7	**UC-**	0.91	0.57	0.22	0.92	0.76	0.31	0.88	
9	**BC+**		0.41	0.29	0.40	0.44	0.18	0.59	0.72
9	**BC-**	0.41		0.06	0.10	0.94	0.52	0.77	0.24
9	**CS+**	0.29	0.06		0.84	0.10	0.03	0.11	0.48
9	**CS-**	0.40	0.10	0.84		0.13	0.04	0.17	0.63
9	**NC+**	0.44	0.94	0.10	0.13		0.62	0.74	0.29
9	**NC-**	0.18	0.52	0.03	0.04	0.62		0.38	0.10
9	**UC+**	0.59	0.77	0.11	0.17	0.74	0.38		0.38
9	**UC-**	0.72	0.24	0.48	0.63	0.29	0.10	0.38	
12	**BC+**		0.34	0.10	0.05	0.00	0.04	0.05	0.25
12	**BC-**	0.34		0.01	0.00	0.00	0.19	0.31	0.84
12	**CS+**	0.10	0.01		0.75	0.00	0.00	0.00	0.01
12	**CS-**	0.05	0.00	0.75		0.00	0.00	0.00	0.00
12	**NC+**	0.00	0.00	0.00	0.00		0.12	0.04	0.01
12	**NC-**	0.04	0.19	0.00	0.00	0.12		0.67	0.27
12	**UC+**	0.05	0.31	0.00	0.00	0.04	0.67		0.42
12	**UC-**	0.25	0.84	0.01	0.00	0.01	0.27	0.42	
16	**BC+**		0.73	0.06	0.00	0.26	0.80	0.54	0.28
16	**BC-**	0.73		0.03	0.00	0.40	0.96	0.34	0.16
16	**CS+**	0.06	0.03		0.16	0.01	0.06	0.20	0.42
16	**CS-**	0.00	0.00	0.16		0.00	0.00	0.01	0.03
16	**NC+**	0.26	0.40	0.01	0.00		0.43	0.10	0.05
16	**NC-**	0.80	0.96	0.06	0.00	0.43		0.44	0.24
16	**UC+**	0.54	0.34	0.20	0.01	0.10	0.44		0.63
16	**UC-**	0.28	0.16	0.42	0.03	0.05	0.24	0.63	
19	**BC+**		0.09	0.33	0.00	0.45	0.56	0.28	0.13
19	**BC-**	0.09		0.01	0.00	0.50	0.38	0.01	0.00
19	**CS+**	0.33	0.01		0.05	0.12	0.16	0.92	0.59
19	**CS-**	0.00	0.00	0.05		0.00	0.00	0.06	0.15
19	**NC+**	0.45	0.50	0.12	0.00		0.87	0.10	0.05
19	**NC-**	0.56	0.38	0.16	0.00	0.87		0.13	0.06
19	**UC+**	0.28	0.01	0.92	0.06	0.10	0.13		0.67
19	**UC-**	0.13	0.00	0.59	0.15	0.05	0.06	0.67	

time	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
22	**BC+**		0.05	0.23	0.01	0.62	0.58	0.22	0.07
22	**BC-**	0.05		0.00	0.00	0.24	0.25	0.00	0.00
22	**CS+**	0.23	0.00		0.09	0.14	0.12	0.98	0.53
22	**CS-**	0.01	0.00	0.09		0.00	0.00	0.09	0.28
22	**NC+**	0.62	0.24	0.14	0.00		0.97	0.13	0.05
22	**NC-**	0.58	0.25	0.12	0.00	0.97		0.11	0.04
22	**UC+**	0.22	0.00	0.98	0.09	0.13	0.11		0.55
22	**UC-**	0.07	0.00	0.53	0.28	0.05	0.04	0.55	
28	**BC+**		0.04	0.35	0.01	0.98	0.56	0.28	0.05
28	**BC-**	0.04		0.00	0.00	0.08	0.23	0.00	0.00
28	**CS+**	0.35	0.00		0.09	0.45	0.17	0.89	0.30
28	**CS-**	0.01	0.00	0.09		0.02	0.00	0.11	0.47
28	**NC+**	0.98	0.08	0.45	0.02		0.59	0.37	0.11
28	**NC-**	0.56	0.23	0.17	0.00	0.59		0.13	0.03
28	**UC+**	0.28	0.00	0.89	0.11	0.37	0.13		0.38
28	**UC-**	0.05	0.00	0.30	0.47	0.11	0.03	0.38	

Appendix VII - pairwise comparisons between each group Sensitivity Analysis (p-values listed)

	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
0	**BC+**		0.33	0.00	0.00	0.01	0.02	0.00	0.00
0	**BC-**	0.33		0.00	0.03	0.09	0.11	0.00	0.00
0	**CS+**	0.00	0.00		0.02	0.03	0.02	0.71	0.48
0	**CS-**	0.00	0.03	0.02		0.86	0.75	0.06	0.11
0	**NC+**	0.01	0.09	0.03	0.86		0.90	0.06	0.11
0	**NC-**	0.02	0.11	0.02	0.75	0.90		0.04	0.08
0	**UC+**	0.00	0.00	0.71	0.06	0.06	0.04		0.75
0	**UC-**	0.00	0.00	0.48	0.11	0.11	0.08	0.75	
1	**BC+**		0.09	0.11	0.00	0.00	0.00	0.00	0.00
1	**BC-**	0.09		0.94	0.05	0.11	0.11	0.04	0.00
1	**CS+**	0.11	0.94		0.05	0.11	0.10	0.04	0.00
1	**CS-**	0.00	0.05	0.05		0.85	0.85	0.88	0.03
1	**NC+**	0.00	0.11	0.11	0.85		1.00	0.76	0.04
1	**NC-**	0.00	0.11	0.10	0.85	1.00		0.75	0.03
1	**UC+**	0.00	0.04	0.04	0.88	0.76	0.75		0.05
1	**UC-**	0.00	0.00	0.00	0.03	0.04	0.03	0.05	
2	**BC+**		0.44	0.43	0.02	0.16	0.11	0.03	0.00
2	**BC-**	0.44		0.98	0.11	0.45	0.35	0.13	0.00
2	**CS+**	0.43	0.98		0.13	0.48	0.37	0.15	0.00
2	**CS-**	0.02	0.11	0.13		0.51	0.62	0.96	0.07
2	**NC+**	0.16	0.45	0.48	0.51		0.88	0.55	0.03
2	**NC-**	0.11	0.35	0.37	0.62	0.88		0.66	0.03
2	**UC+**	0.03	0.13	0.15	0.96	0.55	0.66		0.06
2	**UC-**	0.00	0.00	0.00	0.07	0.03	0.03	0.06	
4	**BC+**		0.42	0.86	0.07	0.35	0.16	0.52	0.01
4	**BC-**	0.42		0.33	0.28	0.80	0.47	0.91	0.04
4	**CS+**	0.86	0.33		0.05	0.28	0.12	0.41	0.00
4	**CS-**	0.07	0.28	0.05		0.48	0.81	0.26	0.35
4	**NC+**	0.35	0.80	0.28	0.48		0.68	0.73	0.13
4	**NC-**	0.16	0.47	0.12	0.81	0.68		0.43	0.27
4	**UC+**	0.52	0.91	0.41	0.26	0.73	0.43		0.04
4	**UC-**	0.01	0.04	0.00	0.35	0.13	0.27	0.04	
7	**BC+**		0.81	0.31	0.84	0.80	0.38	0.67	0.93
7	**BC-**	0.81		0.20	0.66	0.96	0.49	0.51	0.89
7	**CS+**	0.31	0.20		0.42	0.26	0.08	0.57	0.26
7	**CS-**	0.84	0.66	0.42		0.66	0.29	0.82	0.77

	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
7	**NC+**	0.80	0.96	0.26	0.66		0.57	0.53	0.87
7	**NC-**	0.38	0.49	0.08	0.29	0.57		0.22	0.43
7	**UC+**	0.67	0.51	0.57	0.82	0.53	0.22		0.61
7	**UC-**	0.93	0.89	0.26	0.77	0.87	0.43	0.61	
9	**BC+**		0.70	0.30	0.30	0.49	0.26	0.92	0.65
9	**BC-**	0.70		0.15	0.15	0.72	0.42	0.79	0.40
9	**CS+**	0.30	0.15		0.98	0.12	0.05	0.27	0.56
9	**CS-**	0.30	0.15	0.98		0.10	0.04	0.27	0.56
9	**NC+**	0.49	0.72	0.12	0.10		0.70	0.56	0.29
9	**NC-**	0.26	0.42	0.05	0.04	0.70		0.32	0.13
9	**UC+**	0.92	0.79	0.27	0.27	0.56	0.32		0.59
9	**UC-**	0.65	0.40	0.56	0.56	0.29	0.13	0.59	
12	**BC+**		0.62	0.02	0.01	0.00	0.02	0.01	0.11
12	**BC-**	0.62		0.00	0.00	0.00	0.04	0.02	0.25
12	**CS+**	0.02	0.00		0.67	0.00	0.00	0.00	0.00
12	**CS-**	0.01	0.00	0.67		0.00	0.00	0.00	0.00
12	**NC+**	0.00	0.00	0.00	0.00		0.01	0.01	0.00
12	**NC-**	0.02	0.04	0.00	0.00	0.01		0.83	0.32
12	**UC+**	0.01	0.02	0.00	0.00	0.01	0.83		0.19
12	**UC-**	0.11	0.25	0.00	0.00	0.00	0.32	0.19	
16	**BC+**		0.26	0.00	0.00	0.08	0.39	0.07	0.01
16	**BC-**	0.26		0.00	0.00	0.01	0.89	0.42	0.09
16	**CS+**	0.00	0.00		0.01	0.00	0.00	0.03	0.13
16	**CS-**	0.00	0.00	0.01		0.00	0.00	0.00	0.00
16	**NC+**	0.08	0.01	0.00	0.00		0.02	0.00	0.00
16	**NC-**	0.39	0.89	0.00	0.00	0.02		0.40	0.11
16	**UC+**	0.07	0.42	0.03	0.00	0.00	0.40		0.41
16	**UC-**	0.01	0.09	0.13	0.00	0.00	0.11	0.41	
19	**BC+**		0.01	0.00	0.00	0.11	0.90	0.00	0.00
19	**BC-**	0.01		0.00	0.00	0.51	0.02	0.00	0.00
19	**CS+**	0.00	0.00		0.00	0.00	0.01	0.78	0.03
19	**CS-**	0.00	0.00	0.00		0.00	0.00	0.00	0.00
19	**NC+**	0.11	0.51	0.00	0.00		0.11	0.00	0.00
19	**NC-**	0.90	0.02	0.01	0.00	0.11		0.00	0.00
19	**UC+**	0.00	0.00	0.78	0.00	0.00	0.00		0.06
19	**UC-**	0.00	0.00	0.03	0.00	0.00	0.00	0.06	

	Group	BC+	BC-	CS+	CS-	NC+	NC-	UC+	UC-
22	**BC+**		0.00	0.00	0.00	0.45	0.75	0.00	0.00
22	**BC-**	0.00		0.00	0.00	0.02	0.00	0.00	0.00
22	**CS+**	0.00	0.00		0.00	0.00	0.00	0.83	0.01
22	**CS-**	0.00	0.00	0.00		0.00	0.00	0.00	0.04
22	**NC+**	0.45	0.02	0.00	0.00		0.33	0.00	0.00
22	**NC-**	0.75	0.00	0.00	0.00	0.33		0.00	0.00
22	**UC+**	0.00	0.00	0.83	0.00	0.00	0.00		0.02
22	**UC-**	0.00	0.00	0.01	0.04	0.00	0.00	0.02	
28	**BC+**		0.00	0.00	0.00	0.13	0.68	0.00	0.00
28	**BC-**	0.00		0.00	0.00	0.00	0.00	0.00	0.00
28	**CS+**	0.00	0.00		0.00	0.02	0.00	0.09	0.00
28	**CS-**	0.00	0.00	0.00		0.00	0.00	0.00	0.41
28	**NC+**	0.13	0.00	0.02	0.00		0.31	0.00	0.00
28	**NC-**	0.68	0.00	0.00	0.00	0.31		0.00	0.00
28	**UC+**	0.00	0.00	0.09	0.00	0.00	0.00		0.00
28	**UC-**	0.00	0.00	0.00	0.41	0.00	0.00	0.00	

Appendix VIII – Pairwise Post Hoc Test Compared by Group (Original and Sensitivity)

Table 8a. NC+ group comparison Original

Time	BC+	BC-	CS+	CS-	NC-	UC+	UC-
0	x	o	x	o	o	x	o
1	x	o	x	o	o	o	o
2	o	o	o	o	o	o	o
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	x	x	x	x	o	x	x
16	o	o	x	x	o	o	x
19	o	o	o	x	o	o	x
22	o	o	o	x	o	o	x
28	o	o	o	x	o	o	o

x = p<0.50; o= p>0.50

Table 8b. NC+ group comparisons Sensitivity

Time	BC+	BC-	CS+	CS-	NC-	UC+	UC-
0	x	o	x	o	o	o	o
1	x	o	o	o	o	o	x
2	o	o	o	o	o	o	x
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	x	x	x	x	x	x	x
16	o	x	x	x	x	x	x
19	o	o	x	x	o	x	x
22	o	x	x	x	o	x	x
28	o	x	x	x	o	x	x

x = p<0.50; o= p>0.50

Table 9a. NC- group comparison - Original							
Time	BC+	BC-	CS+	CS-	NC+	UC+	UC-
0	x	o	x	o	o	x	o
1	x	o	x	o	o	o	o
2	o	o	o	o	o	o	o
4	o	o	x	o	o	o	o
7	o	o	x	o	o	o	o
9	o	o	x	x	o	o	o
12	x	o	x	x	o	o	o
16	o	o	o	x	o	o	o
19	o	o	o	x	o	o	o
22	o	o	o	x	o	o	x
28	o	o	o	x	o	o	x
x = p<0.50; o= p>0.50							

Table 9b. NC- group comparison - Sensitivity							
Time	BC+	BC-	CS+	CS-	NC+	UC+	UC-
0	x	o	x	o	o	x	o
1	x	o	o	o	o	o	x
2	o	o	o	o	o	o	x
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	x	x	o	o	o
12	x	x	x	x	x	o	o
16	o	o	x	x	x	o	o
19	o	x	x	x	o	x	x
22	o	x	x	x	o	x	x
28	o	x	x	x	o	x	x
x = p<0.50; o= p>0.50							

Table 10a. CS+ group comparison							
Time	BC+	BC-	CS-	NC+	NC-	UC+	UC-
0	x	x	x	x	x	o	o
1	o	o	x	x	x	x	x
2	o	o	x	o	o	x	x
4	o	o	x	o	x	x	x
7	o	o	o	o	x	o	o
9	o	o	o	o	x	o	o
12	o	x	o	x	x	x	x
16	o	x	o	x	o	o	o
19	o	x	x	o	o	o	o
22	o	x	o	o	o	o	o
28	o	x	o	o	o	o	o
x = p<0.50; o= p>0.50							

Table 10b. CS+ group comparison - Sensitivity							
Time	BC+	BC-	CS-	NC+	NC-	UC+	UC-
0	x	x	x	x	x	o	o
1	o	o	x	o	o	x	x
2	o	o	o	o	o	o	x
4	o	o	o	o	o	o	x
7	o	o	o	o	o	o	o
9	o	o	o	o	x	o	o
12	x	x	o	x	x	x	x
16	x	x	x	x	x	x	o
19	x	x	x	x	x	o	x
22	x	x	x	x	x	o	x
28	x	x	x	x	x	o	x
x = p<0.50; o= p>0.50							

Table 11a. CS- group comparison

Time	BC+	BC-	CS+	NC+	NC-	UC+	UC-
0	x	x	x	o	o	x	o
1	x	o	x	o	o	o	o
2	o	o	x	o	o	o	o
4	o	o	x	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	x	o	o
12	o	x	o	x	x	x	x
16	x	x	o	x	x	x	x
19	x	x	x	x	x	o	o
22	x	x	o	x	x	o	o
28	x	x	o	x	x	o	o

x = p<0.50; o= p>0.50

Table 11b. CS- group comparison – Sensitivity Analysis

Time	BC+	BC-	CS+	NC+	NC-	UC+	UC-
0	x	x	x	o	o	o	o
1	x	x	x	o	o	o	x
2	x	o	o	o	o	o	o
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	x	o	o
12	x	x	o	x	x	x	x
16	x	x	x	x	x	x	x
19	x	x	x	x	x	x	x
22	x	x	x	x	x	x	x
28	x	x	x	x	x	x	o

x = p<0.50; o= p>0.50

Table 12. UC+ group comparison							
Time	BC+	BC-	CS+	CS-	NC+	NC-	UC-
0	x	x	o	x	x	x	o
1	x	x	x	o	o	o	o
2	x	o	x	o	o	o	o
4	o	o	x	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	o	x	x	x	o	o
16	o	o	o	x	o	o	o
19	o	x	o	o	o	o	o
22	o	x	o	o	o	o	o
28	o	x	o	o	o	o	o
							x = p<0.50; o= p>0.50

Table 12b. UC+ group comparison - Sensitivity							
Time	BC+	BC-	CS+	CS-	NC+	NC-	UC-
0	x	x	o	o	o	x	o
1	x	x	x	o	o	o	x
2	x	o	o	o	o	o	o
4	o	o	o	o	o	o	x
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	x	x	x	x	x	o	o
16	o	o	x	x	x	o	o
19	x	x	o	x	x	x	o
22	x	x	o	x	x	x	x
28	x	x	o	x	x	x	x
							x = p<0.50; o= p>0.50

Table 13a. UC- comparison group							
Time	BC+	BC-	CS+	CS-	NC+	NC-	UC+
0	x	x	o	o	o	o	o
1	x	x	x	o	o	o	o
2	x	x	x	o	o	o	o
4	o	o	x	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	o	x	x	x	o	o
16	o	o	o	x	x	o	o
19	o	x	o	o	x	o	o
22	o	x	o	o	x	x	o
28	o	x	o	o	o	x	o
x = p<0.50; o= p>0.50							

Table 13b. UC- comparison group - Sensitivity							
Time	BC+	BC-	CS+	CS-	NC+	NC-	UC+
0	x	x	o	o	o	o	o
1	x	x	x	x	x	x	x
2	x	x	x	o	x	x	o
4	x	x	x	o	o	o	x
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	o	x	x	x	o	o
16	x	o	o	x	x	o	o
19	x	x	x	x	x	x	o
22	x	x	x	x	x	x	x
28	x	x	x	o	x	x	x
x = p<0.50; o= p>0.50							

Table 14a. BC+ comparison group							
Time	BC-	CS+	CS-	NC+	NC-	UC+	UC-
0	o	x	x	x	x	x	x
1	o	o	x	x	x	x	x
2	o	o	o	o	o	x	x
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	o	o	x	x	o	o
16	o	o	x	o	o	o	o
19	o	o	x	o	o	o	o
22	o	o	x	o	o	o	o
28	x	o	x	o	o	o	o
x = p<0.50; o= p>0.50							

Table 14b. BC+ comparison group - Sensitivity							
Time	BC-	CS+	CS-	NC+	NC-	UC+	UC-
0	o	x	x	x	x	x	x
1	o	o	x	x	x	x	x
2	o	o	x	o	o	x	x
4	o	o	o	o	o	o	x
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	x	x	x	x	x	o
16	o	x	x	o	o	o	x
19	x	x	x	o	o	x	x
22	x	x	x	o	o	x	x
28	x	x	x	o	o	x	x
x = p<0.50; o= p>0.50							

Table 15a. BC- comparison groups

Time	BC+	CS+	CS-	NC+	NC-	UC+	UC-
0	o	x	x	o	o	x	x
1	o	o	o	o	o	x	x
2	o	o	o	o	o	o	x
4	o	o	o	o	o	o	o
7	o	o	o	o	o	o	o
9	o	o	o	o	o	o	o
12	o	x	x	x	o	o	o
16	o	x	x	o	o	o	o
19	o	x	x	o	o	x	x
22	o	x	x	o	o	x	x
28	x	x	x	o	o	x	x

x = p<0.50; o= p>0.50

Table 15b. BC- comparison groups - Sensitivity

Time	BC+	CS+	CS-	NC+	NC-	UC+	UC-
0	o	x	x	o	o	x	x
1	o	o	x	o	o	x	x
2	o	o	o	o	o	o	x
4	o	o	o	o	o	o	x
7	o	o	o	o	o	o	o
9	o	o	x	o	o	o	o
12	o	x	x	x	x	x	o
16	o	x	x	x	o	o	o
19	x	x	x	o	x	x	x
22	x	x	x	x	x	x	x
28	x	x	x	x	x	x	x

x = p<0.50; o= p>0.50