Overcoming the Adverse Effects Related to the Use of Smear Layer Removal Agents: Phosphoric Acid as Etchant and EDTA as Chelating Agent

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DEDICATION

There are people in everyone’s life who make success both possible and rewarding. I dedicate this work to my family, who despite the distance were very supportive and encouraging.

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LIST OF ABBREVIATIONS

Term	Abbreviation
Alkaline phosphatase	ALP
Calcium	Ca
Chlorhexidine	CHX
Ethylenediaminetetraacetic acid	EDTA
2-Hydroxyethylmethacrylate	HEMA
Matrix metalloproteinases	MMPs
Megapascal	MPa
Microtensile bond strength	μTBS
N-acetylcysteine	NAC
Non-Protein Thiols	NPSH
Oxidized glutathione	GSSG
Phosphoric acid	PA
Phytic acid	IP6
Reduced glutathione	GSH
Sodium hypochlorite	NaOCl
Zinc	Zn

SUMMARY

OVERCOMING THE ADVERSE EFFECTS RELATED TO THE USE OF SMEAR LAYER REMOVAL AGENTS: PHOSPHORIC ACID AS ETCHANT AND EDTA AS CHELATING AGENT

Done by: Mohannad Issa Michael Nassar

Introduction PA and EDTA are two agents that are clinically used to remove the smear layer created on dentin surfaces after various dental treatments. Despite the development of resin adhesive systems and the advent of self-etch adhesives, PA is still the etching agent of choice in etch-and-rinse adhesives. It removes the inorganic part of the smear layer exposing the collagen network that would be later infiltrated with resin to form the hybrid layer. EDTA is the most commonly used chelating agent in root canal treatment that is used in a concentration of 17% and an application time of 1-5 minutes. It is applied as a final rinse before obturation to remove the smear layer created on root canal dentin surfaces during instrumentation as NaOCl, the main irrigant in root canal treatment, is not effective to completely remove the smear layer. Regardless of the popularity of PA and EDTA, they are not without their drawbacks. PA etching results in the formation of fragile dentinal collagen network that are denuded of hydroxyapatite, thus susceptible to collapse resulting in poor infiltration of resin adhesive. Etching with PA also results in the activation of host-derived dentinal MMPs which could degrade dentinal collagen. The two aforementioned effects contribute to compromised resin-dentin bonding. Moreover; PA application increases dentin permeability, thus allowing resin monomers especially HEMA to leach into the pulp enhanced by the latter low molecular weight and high hydrophilicity. HEMA is known to negatively affect the pulpal cells causing apoptotic cell death. EDTA, a synthetic and not readily biodegradable material which is considered as a pollutant, can show some cytotoxic effect on osteoblast cells if extruded beyond the apical foramen thereby hampering the healing of the periradicular area after root canal treatment. Considering these facts the search for a more biocompatible material to replace EDTA as a chelating agent is still going on. NPSH, namely GSH and NAC, are a group of antioxidants that are crucial for detoxification of a number of cytotoxic products especially resin monomers. Several mechanisms have been proposed for this detoxification action; however, direct interaction between the cysteine residue in these

antioxidants and resin monomers was speculated to be the main mechanism. They also have the ability to inhibit MMPs through binding with MMPs active-site Zn. IP6 is a naturally occurring substance in plants and mammalian cells. It can be extracted with a low price from rice bran. This agent is highly negatively charged therefore it has the ability to chelate with multivalent cations such as Ca^{+2}. **Aims** The purpose of these studies were to evaluate the detoxification effect of NPSH on HEMA cytotoxicity on pulpal-like cells and their inhibitory effect on dentinal MMPs that are activated by the use of PA. Furthermore; we evaluated the effect of IP6 used as etchant and chelating agent on the bond strength of resin adhesives to dentin and smear layer removal from flat dentinal surfaces and instrumented root canals. Finally, IP6 effect on the viability of pulpal-like cells and the viability and ALP activity of osteoblast-like cells were assessed. **Methodology** Dentin discs and dentin powder, used in these studies, were obtained from the coronal part of sound human molar teeth while the roots were obtained from human anterior teeth. Pulpal-like cells (RPC-C2A) established from dental pulp of rat incisors and osteoblast-like cells (MC3T3-E1) established from mouse calvaria were used in the conducted studies. The effect of different concentrations of GSH on the bond strength of etch-and-rinse resin adhesive to dentin discs and the effect of GSH and NAC on the detoxification of the toxicity of HEMA on the cultured pulpal-like cells were performed. Dentin powder was used to study the effect of PA on the activation of MMPs and to assess the ability of NPSH to deactivate these MMPs. The effect of IP6 in removing the smear layer from flat dentinal discs and instrumented root canals were evaluated and compared to PA and EDTA. The effect of IP6 on the adhesion of resin to dentin was performed and its effect on pulpal- and osteoblast-like cells was also evaluated. **Results** GSH abolished the toxic effect of HEMA on pulpal-like cells and its effect was comparable to NAC. PA activated dentinal MMPs while the use of NPSH resulted in their inhibition. The use of GSH on etched dentin had no immediate effect on resin-dentin bond strength. The use of IP6 as etchant resulted in enhanced resin-dentin bond strength, clean dentinal surfaces and it showed minimal effect on pulpal-like cells when compared to PA. IP6 was effective in removing root canal smear layer while being biocompatible to osteoblast-like cells. **Conclusions** The used NPSH showed promising results to be used as HEMA detoxification agents and dentinal MMP inhibitors. IP6 has the potential to replace PA and EDTA as etchant and chelating agent, respectively.

SUMMARY (Japanese)

課題名

象牙質接着時のリン酸エッチング、EDTA キレート根管洗浄剤の問題点と対処法

序論：リン酸（PA）、エチレンジアミン 四酢酸（EDTA）は保存歯科治療でスメア層除去のために使われる薬品である。PA は主にエッチアンドリンス接着システムに使われ、スメア層除去し、有機質であるコラーゲンを露出させ、レジンモノマーを浸透させる効果がある。EDTA は歯内治療の際に使われ、そのキレート作用により根管壁のカルシウムを溶出させ、スメア層を除去し、根充前の最終洗浄剤と使用されている。PA と EDTA は臨床で汎用させているが、欠点もある。PA はコラーゲンを露出させ、変性させる恐れがあり、さらに、内在性のマトリックスメタロプロテアーゼタンパク質分解酵素(Matrix metalloproteinases、MMPs)を活性化させるとの報告がある。また、PA 処理させた象牙質はメタクリル酸 2-ヒドロキシエチル(HEMA)等の低分子のモノマーが浸透しやすく、その化学的刺激により歯髄への影響が懸念される。EDTA も本来細胞毒性の危険がある薬品で、根尖から滲出すれば、周辺組織への影響が懸念されている。非タンパク質チオールのグルタチオンや N-アセチルシステインは、レジンモノマーからの有害物質から細胞を保護する解毒的役割を有し、MMPs の不活性化作用もあるといわれている。また、フィチン酸 (IP6) は植物、哺乳動物などの組織に自然に存在し、（-）の電荷によりキレート作用が強く、多くの金属イオンを強く結合する特徴がある。

目的：研究の目的は、非タンパク質チオールによる、歯髄細胞毒性モノマーである HEMA の解毒作用、PA より活性化された MMPs の抑制作用を評価することである。さらに、IP6 によるスメア層除去の効果と、象牙質接着強さに及ぼす効果、また根管洗浄剤として使用した場合の有効性、そして歯髄細胞様細胞、及び骨芽細胞様細胞への毒性を調べた。

方法：円盤状また粉末の象牙質試料を健全な臼歯から、歯根は前歯部から準備した。歯髄様細胞は、ラットの前歯、骨芽様細胞はマウス頭蓋骨由来を使用した。濃度の異なるグルタチオンで歯面処理した場合の、歯科接着剤の象牙質接着強さと、グルタチオンや N-アセチルシステインの HEMA の解毒作用を歯髄細胞様細胞を使い MTT assay にて比較した。また粉末象牙質を使い、PA の MMPs 活性化作用、その活性化した MMPs のグルタチオンや N-アセチルシステインによる抑制効果を検討した。IP6 のエッチング効果は、象牙質接着時を想定し平面な象牙質盤上のスメア層、また根管洗浄を想定し根管内壁のスメア層を使い,PA と EDTA の効果と比較した。IP6 の歯髄細胞様細胞と骨芽細胞様細胞への毒性を MTT assay 測定し、評価した。

結果：グルタチオンは N-アセチルシステインと同様、歯髄細胞様細胞への HEMA 毒性を軽減させた。PA は MMPs を活性化させたが、グルタチオンと N-アセチルシステインはそれを不活性化させた。グルタチオンを、エッチングさせた象牙質に塗布させた場合、象牙質接着強さは影響されなかった。PA と比較し、IP6 によるエッチングは接着強さを向上させ、歯髄細胞様細胞への毒性も見られなかった。IP6 のスメア層除去効果は根管洗浄剤としても、有効で、さらに骨芽細胞様細胞への影響もなかった。

結論：グルタチオンと N-アセチルシステインは HEMA 毒性を軽減し、MMPs 抑制効果も見られた。IP6 は、PA と EDTA と比べ、より有効なスメア層除去剤で、生体に安全であることから、PA、EDTA に代わる薬剤であると示唆できた。

ABSTRACT I

The Effect of Glutathione on 2-Hydroxyethylmethacrylate Cytotoxicity and on Resin-Dentin Bond Strength

Aim This study evaluated the influence of GSH application on HEMA cytotoxicity on rat pulpal cells, and evaluated the effect of etched-dentin treatment with GSH on the immediate μTBS of etch-and-rinse adhesive. **Methodology** The cytotoxicity of 10 mM HEMA, 10 mM HEMA+1 mM GSH, 10 mM HEMA+5 mM GSH and 10 mM HEMA+10 mM GSH was compared (6 hours and 24 hours). Cells viability was measured by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, followed by morphological observation of cells. Etched dentin surfaces were rinsed and treated with one of the following solutions: 2% GSH, 5% GSH or 10% GSH, bonded with Adper Single Bond Plus (3M, ESPE) and restored with resin composite. The control group received no GSH treatment. After 1 day of water-storage at 37 ºC, the specimens were subjected to μTBS testing. Cytotoxicity and μTBS data were analyzed by one-way ANOVA and Tukey post-hoc tests ($P < 0.05$). **Results** There were statistically significant differences among the groups. HEMA elicited remarkable toxic effect. 10 mM GSH prevented HEMA-induced damage at both exposure times, whereas 5 mM GSH lost its protective effect at 24-hour exposure time and 1 mM GSH showed no protective effect at both exposure times. GSH had no significant effect on the immediate μTBS; however, 5% GSH resulted in higher bond strength value when compared to 10% GSH ($P = 0.003$). **Conclusion** Controlled concentrations of GSH had a protective effect against HEMA cytotoxicity. GSH showed neither positive nor negative influence on μTBS.

ABSTRACT II

The Inhibition Effect of Non-Protein Thiols on Dentinal Matrix Metalloproteinase Activity and HEMA Cytotoxicity

Aims PA etching used in etch-and-rinse adhesives is known to activate host-derived dentinal MMPs and increase dentin permeability. These two phenomena will result, respectively; in degradation of dentin-adhesive bond and leaching of some monomers especially HEMA into the pulp that would negatively affect the viability of pulpal cells. This study is the first to investigate the inhibitory effect of NPSH; namely GSH) and NAC on dentinal MMPs and compare their effects on HEMA cytotoxicity. **Methodology** Dentin powder was prepared from human teeth, demineralized with 1% PA and then treated with 2% GSH, 2% NAC or 2% CHX. Zymographic analysis of extracted proteins was performed. To evaluate the effect of GSH, NAC and CHX on HEMA cytotoxicity, solutions of these compounds were prepared with or without HEMA and rat pulpal cells were treated with the tested solutions for (6 hours and 24 hours). Cells viability was measured by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxicity data were analyzed by one-way ANOVA and Tukey post-hoc tests ($P < 0.05$). **Results** The inhibitory effect of GSH and NAC on dentinal MMPs was confirmed. GSH showed similar effectiveness to NAC regarding HEMA cytotoxicity inhibition. **Conclusion** NPSH were effective to inhibit dentinal MMPs and HEMA cytotoxicity. The tested properties of NPSH provide promising clinical use of these agents which would enhance dentin-bond durability and decrease post-operative sensitivity.

ABSTRACT III

The Effect of Phytic Acid Used as Etchant on the Bond Strength, Smear Layer and Pulpal Cells

Aim This study aimed to evaluate the influence of IP6 used as etchant on resin-dentin bond strength, smear layer removal and the effect on pulpal cells. Flat dentin surfaces with smear layer were etched with 1% IP6 for 1 minute, 30 seconds or 15 seconds; in the control group 37% PA was used. **Methodology** Dentin surfaces were rinsed, blot-dried and bonded with an etch-and-rinse adhesive, followed by composite build-ups. The specimens were subjected to tensile testing after 24-hour water storage at 37°C, and failure modes were determined under scanning electron microscope. The effectiveness of IP6 to remove the smear layer was also observed under scanning electron microscope. To evaluate the effect on pulpal cells, solutions of 0.1% IP6, 0.01% IP6, 3.7% PA and 0.37% PA were prepared and rat pulpal cells were treated with these solutions for 6 hours and 24 hours. Cells' viability was measured by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results** The results demonstrated that all application times of IP6 produced bond strength values that were significantly higher than the control. IP6 effectively removed the smear layer and plugs, thus exposing the collagen network. IP6 had a minimal effect on pulpal cells, whereas PA resulted in marked decrease in their viability. **Conclusion** IP6 shows the potential to replace PA as an etchant in etch-and-rinse adhesive systems as it was able to etch dentinal surfaces and produced high resin-dentin bond strength with being biocompatible to pulpal cells.

ABSTRACT IV

Phytic Acid: An Alternative Root Canal Chelating Agent

Aims This study were designed to investigate the effect of IP6, used as a final rinse, on the surface of instrumented root canals and smear layered-flat dentin surfaces and evaluate its effect on the viability, ALP activity and morphology of osteoblastic-like cells. Moreover, we assessed IP6 effect on resin adhesion to NaOCl-treated dentin. **Methodology** The universally accepted chelating agent EDTA was used as the control in all conducted experiments. Root canals of human canines were instrumented and irrigated with 5% NaOCl followed by a final rinse of either 17% EDTA (1 minute), 1% IP6 (1 minute or 30 seconds) or distilled water. NaOCl-treated dentin surfaces were also treated with 17% EDTA (1 minute), 1% IP6 (1 minute or 30 seconds) or distilled water. The presence or absence of smear layer was evaluated with a scanning electron microscope. Cell viability and ALP assays were performed to evaluate the effect of IP6 and EDTA on cultured osteoblastic-like cells. To study the effect on resin-dentin adhesion the μTBS test was performed using one-step self-etch adhesive on NaOCl-treated dentin discs that were rinsed with either 17% EDTA (1 minute) or IP6 (1 minute or 30 seconds). We assessed the reaction between these reagents and NaOCl by mixing 1 ml of 5% NaOCl with either 17% EDTA or 1% IP6 and recoding changes in temperature, pH, bubble formation and the effect of the mixtures on the color of hematotropin-stained paper. **Results** The results demonstrated the ability of IP6 to remove the smear layer from instrumented root canals and flat dentin surfaces treated with NaOCl. When compared to EDTA, IP6 was less cytotoxic and did not affect the differentiation of osteoblastic-like cells. IP6 reversed NaOCl-negative effect on resin-dentin adhesion. The reaction between NaOCl and EDTA was exothermic with bubble formation and this mixture did not have a bleaching effect on the stained paper while the reaction with IP6 was non-exothermic, showed no bubble formation and changed the color of the stained paper to white. **Conclusion** We concluded that IP6 is an effective chelating agent with osteoblastic-like cells biocompatibility, a positive influence on the resin adhesion to NaOCl-treated dentin and less reactivity with NaOCl, thus having the potential to replace EDTA as a chelating agent.

CHAPTER ONE: INTRODUCTION

1. Phosphoric Acid

Etch-and-rinse adhesive systems are considered as the oldest generation of resin bond systems that are still in use. In these systems, a specific acid-etch step is required to expose collagen fibrils that will be infiltrated with resin adhesive forming the hybrid layer (Pashley *et al.,* 2011; Van Meerbeek *et al.* 2003). PA used at a concentration of 30–40% and applied for 15 seconds, has historically been the gold standard etchant (Gardner and Hobson, 2001). Although more effective enamel bonding is achieved through etching with PA (Frankenberger *et al*., 2008), nowadays dentin etching with PA is considered too aggressive to dentin (Van Meerbeek *et al.,* 2011). Application of PA to dentin results in the exposure of collagen fibrils that are totally devoid of hydroxyapatite (Van Meerbeek *et al.,* 1996; De Munk *et al.,* 2003). These fragile collagen fibrils are susceptible to collapse thus resulting in imperfect resin infiltration (Prati *et al*., 1999; El Feninat *et al.,* 2001). This poor impregnation of collagen could result in low immediate bond strength (Nakajima *et al.,* 2002) and postoperative sensitivity (Chersoni *et al.,* 2004). Moreover; incompletely infiltrated collagen fibrils are susceptible to degradation by host-derived MMPs, which could jeopardize the long-term durability of resin-dentin bond (Pashley *et al.,* 2004). MMPs are a class of Zn^{+2} and Ca^{+2} dependent enzymes that have the ability to hydrolyze components of the extracellular matrix such as collagen (Visse and Nagase, 2003). PA-treated dentin has rapid collagen degradation due to the activation of dentinal MMPs. However, the use of MMPs' inhibitors such as CHX and doxycycline results in the inhibition of this collagen degradation (Osorio *et al.,* 2011a). Amongst all MMPs in dentin, MMP-2 and MMP-9 along with their inhibitors have gained particular interest (Niu *et al.,* 2011). Previous attempts have been

made to evaluate other etching agents, such as maleic acid, citric acid (Breschi *et al.,* 2002) and EDTA (Sauro *et al.,* 2010). However, PA is still the most commonly used etchant in etch-and-rinse adhesives. Despite the popularity of this etching agent, there is a paucity of literature that adequately shows its effect on pulpal cells.

Another drawback of PA use is the increase in dentin permeability (Pashley, 1992). HEMA is an important constituent of etch-and-rinse adhesives. HEMA is used as a comonomer or solvent with other resin monomers in dentin adhesives to improve their hydrophilicity and promote adhesion to dental substrates (Van Landuyt *et al*., 2007). HEMA also prevents phase separation in solutions containing hydrophilic and hydrophobic resin components (Van Landuyt *et al*., 2005), minimizes collagen collapse and increases dentin wettability (Hitmi *et al.,* 2002). Together, these attributes are responsible for increases in resin-dentin bond strength of HEMA-containing adhesives (Van Landuyt *et al*., 2007). Nevertheless, HEMA could be detected in aqueous extracts of polymerized dentin adhesives even after thorough light-curing (Mantellini *et al*., 2003). Because of its small molecular weight and high hydrophilicity, HEMA can diffuse through dentin toward the dental pulp (Gerzina and Hume, 1995). The amount of HEMA reaching the pulp is considered safe as long as the remaining dentin thickness is more than 1 mm (Cetinguc *et al.,* 2007). The risk of HEMA-induced cytotoxicity increases with decreased dentin thickness, incomplete polymerization and extended exposure time (Bouillaguet *et al.,* 1996). The amount of HEMA reaching the pulp increases with PA etching (Rathke *et al.,* 2007) where HEMA cytotoxicity can adversely affect the pulpal cells (About *et al.,* 2002; Chang *et al.,* 2005). A high concentration of HEMA adversely affects the differentiation of pulpal cells into odontoblast (About *et al*., 2002) and induces apoptotic cell death (Becher *et al.,* 2006). The mechanisms of HEMA cytotoxicity are mainly related to glutathione depletion and reactive

oxygen species production (Chang *et al.,* 2005; Huang *et al*., 2010). Thus, the cytotoxicity of HEMA must be lowered to avoid its detrimental effects on pulpal cells, especially when resin adhesives are applied on deep cavities that are etched with PA.

2. Ethylenediaminetetraacetic Acid

In endodontic there is always a need for chemo-mechanical debridement (Marending *et al*., 2007). Mechanical debridement results in the formation of smear layer on root canal surfaces. Removal of the smear layer before obturation is still a matter of debate; nevertheless, the literature takes side on its removal (Hülsmann *et al*., 2003). The organic components of the smear layer might be a substrate for the bacteria. Not only do bacteria exist inside the smear layer but also grow and multiply (Pashley, 1984), and it can also penetrate into the dentinal tubules up to a depth of 150 μm (Sen *et al.,* 1995). Keeping the smear layer in place would hinder the penetration of intracanal disinfectants into the dentinal tubules (Örstavik and Haapasalo, 1990) and affect the adhesion and penetration of root canal sealers and filling materials which would compromise the seal (White *et al.,* 1984; Kennedy *et al.,* 1986; White *et al.,* 1987; Saunders and Saunders, 1992; Oksan *et al.,* 1993; Economides *et al.,* 1999; Shahravan *et al.,* 2007).

NaOCl is the main irrigant used during root canal treatment due to its many wanted properties (Zehnder, 2006); however, it cannot effectively remove the smear layer (Torabinejad *et al.,* 2003). EDTA is a widely-used abbreviation for the chemical compound ethylenediaminetetraacetic acid which is a chelating agent with the formula $(HO_2CCH2)_2NCH_2CH_2$-$N(CH_2CO2H)_2$ (Hulsmann *et al.,* 2003). Many different fields involve the use of EDTA; it is added to various cosmetics in concentrations less than 2% to improve

products stability (Lanigan andYamarik, 2002), while in medicine it is used in chelation therapy for mercury (Blanusa *et al.,* 2005) and lead poisoning (Llobet *et al.,* 1990). EDTA has been the most commonly used material for the purpose of smear layer removal in root canal treatment since 1957 (Nygaard-Ostby, 1957) in a concentration of 17% and an application time of 1-5 minutes (Calt and Serper, 2002). A survey was conducted in the United States in 2001 about smear layer removal, this survey showed that about 50% of endodontists were applying this practice at their clinics with about two-thirds of them using EDTA which was reported to be the preferred material used to remove this layer (Moss *et al.,* 2001).

The chelation action of EDTA is needed during root canal treatment to remove the smear layer (McComb and Smith, 1975), prepare dentinal walls for better adhesion of the filling materials (White *et al.,* 1987; Calt and Serper, 1999; Villegas *et al.,* 2002), enhance the action of intracanal medicament by increasing the permeability of dentin (Hampson and Atkinson, 1964) and negotiate narrow and calcified canals (Stock and Nehammer, 1985; Stewart, 1995). It is also used as a lubricant during root canal preparation to reduce the risk of instrument separation especially with the nickel-titanium rotary files (Hulsmann *et al.,* 2003). This material is available in a liquid and gel formulae (Hulsmann *et al.,* 2003). It forms a stable complex with Ca^{+2} resulting in the release of protons which decrease the pH of the environment (Seidberg and Schilder, 1974), so its action is said to be self-limiting due to the loss of efficiency in acidic media (Nygaard-Ostby, 1957; Seidberg and Schilder, 1974). The demineralizing effect of EDTA has been reported to be affected by concentration, pH, root canal length, penetration inside the root canal, dentin hardness and time of application of the material (Cury *et al.,* 1981; Baumgartner and Mader 1987; Calvo Perez *et al.,* 1989; Serper and Calt, 2002). The effect of pH has been investigated and it has been shown to play a major role in determining the

demineralizing effect of EDTA. At neutral pH (7.5) better action of EDTA was observed than alkaline one (9.0) (Serper and Calt, 2002). No alteration in the pH of the solutions, used in the study of Serper and Calt, was detected during the observation period which lasts up to 15 minutes; this was attributed to the large volume of EDTA solution used in their study (Serper and Calt, 2002), while in the study of Calvo *et al.* the pH decreased significantly during the demineralization process (Calvo *et al.,* 1989). The higher the number of the ionized molecules of EDTA in a solution, the more effective is the chelation. A higher ratio of ionized to non-ionized molecules can be achieved in solutions with high pH (above 10.3) but the availability of Ca^{+2} from hydroxyapatite for chelation decreases because of the increased number of hydroxyl groups which would retard the dissociation of hydroxyapatite. In other words, at lower pHs the Ca^{+2} will be more available for chelation but the efficacy of EDTA will be less, thus the optimum pH for EDTA solutions for effective chelation would be in the range of 6-10 (O'Connell *et al.,* 2000). There is no consensus on the duration of EDTA application for optimal results (Hulsmann *et al.,* 2003). A 15-minute application time has been reported to have the best cleaning effect (Goldberg and Speilberg, 1982). However, many studies showed that a time of 1-5 minutes is sufficient (Cergneux *et al.,* 1987; Meryon *et al.,* 1987; Calt and Serper, 2000).

EDTA is most commonly synthesized on an industrial scale from ethylenediamine, formaldehyde and sodium cyanide. This method results in the formation of impurities that are detrimental to most applications of this chelating agent (Elvers, 2014). This synthetic persistent material is being overused and considered as one of the major organic pollutants discharged in water (Sillanpaa, 1997). EDTA has been found to be both cytotoxic and weakly genotoxic in laboratory animals. Oral exposures have been noted to cause reproductive and developmental effects. It was also found that both dermal and inhalation exposures to EDTA in most cosmetic

formulations would produce systemic effects below those seen to be toxic in oral dosing studies (Lanigan and Yamarik, 2002). Due to the possibility of introducing EDTA and other chelating agents into the periapical tissues during root canal treatment, its effect has been studied with controversial results. In 1957, Nygaard-Ostby investigated the effect of 15% EDTA solution on human periapical tissue as well as on pulpal tissue under clinical conditions. EDTA was found to be biocompatible when used clinically (Nygaard-Ostby, 1957). Lasala stated that EDTA has no deleterious effects and its extrusion into the periapical tissue caused only decalcifying effect on periapical bone that was reversed in 3 to 4 days (Lasala, 1992). Segura *et al*. studied the effect of EDTA on macrophages, and it appeared that EDTA, even in low concentrations, modified the inflammatory reactions in the periapical tissues through the inhibition of vasoactive intestinal peptide binding to macrophage membranes (Segura *et al*., 1996). This neuropeptide, found in peptidergic intrapulpal and periapical nerve fibers, modulates the function of macrophages and lymphocyte so it is considered as a connection between the nervous and immune system in the pulp and periapical tissues. In 1997, the effect of EDTA on the adherence capacity of macrophages was studied. EDTA, in concentrations lower than that used in root canal treatment, inhibited the macrophage capacity of adherence which may affect phagocytosis and antigen presentation functions of these cells (Segura *et al.,* 1997). Based on these studies, leakage of EDTA into the periapical tissue not only causes decalcification of periapical bone but also has other effects on the immune system, thus its extrusion should be avoided (Segura *et al*., 1996; Segura *et al*., 1997; Segura-Egea *et al*., 2003). Considering these facts, an alternative agent for smear layer removal in root canal treatment is warranted and the search for more biocompatible material to replace EDTA is still going on.

3. Non-Protein Thiols

NPSH is a term that includes all low molecular weight thiol-compounds containing a sulfhydryl group (-SH) in their structure (Mulier *et al.,* 1998). NPSH are considered as antioxidants that work through a variety of mechanisms such as metal chelators and radical quenchers (Deneke, 2000). Glutathione, a NPSH, is the most prevalent and most important intracellular thiol-disulfide redox buffer in mammalian cells. It exits in two forms; GSH and GSSG. GSH, the active form, is a water soluble tripeptide containing cysteine, glutamic acid and glycine (Meister and Anderson, 1983; Pompella *et al*. 2003). GSH contributes to around 90% of the intracellular NPSH. The remaining 10% is made up of other small thiol compounds (Jacobson *et al*., 1990). The thiol group in cysteine is a potent reducing agent, rendering GSH as an essential antioxidant in the detoxification of a variety of electrophilic compounds and peroxides (Townsend *et al.,* 2003). It is found in micromolar (µM) concentrations in body fluids and in millimolar (mM) concentrations in tissue. The GSH:GSSG ratio is indicative of oxidative stress and cellular health (Owen and Butterfield, 2010). It was reported that the GSH:GSSG ratio in the plasma is an indicator of aging (Erden-Inal *et al.,* 2002). A recent study showed that the GSH:GSSG ratio in saliva is lower in patients with periodontitis. However, it was restored to normal levels upon therapy (Grant *et al.,* 2010). Despite the high concentration of glutathione in gingival crevicular fluid, the source of glutathione in its two forms in saliva is yet to be investigated (Chapple *et al.,* 2002). Salivary glutathione levels may be an index of increased levels of oxidative stress and an indication of increased risk of oral diseases (Almadori *et al.,* 2007).

NAC is the most bio-available precursor of glutathione (Gross *et al.,* 1993) that can be readily hydrolyzed to cysteine thus increasing intracellualr GSH levels (Sochman, 2002). NAC

has many medicinal applications; it is the treatment of choice for acetaminophen poisoning (Woodhead *et al.,* 2012) and considered as a classical mucolytic agent (Sadowska *et al.,* 2006). The application of NAC has been found to reverse the cytotoxicity and anti-differentiation effects of dentin adhesives (Paranjpe *et al.*, 2007a,b; Kim *et al*., 2010a), via an adduct formation between the cysteine group of NAC and HEMA (Nocca *et al*., 2010) or via enhancement of intracellular levels of GSH (Krifka *et al.,* 2012). Recently adduct formation between HEMA and intracellular GSH has been also reported (Nocca *et al.,* 2011; Samuelsen *et al.,* 2011).

Interestingly both GSH and NAC have an inhibitory effect on the activation and function of MMP-9 and MMP-2 (Upadhya and Strasberg, 2000; Emara and Cheung, 2006; Pei *et al.,* 2006) the postulated mechanism for this inhibition is the binding between the thiol group and Zn^{+2} (Pei et *al.,* 2006; Koch *et al.,* 2009).

According to these findings it is hypothesised that the application of GSH can act as an agent that prevents HEMA toxicity in the same manner as NAC and we postulate that the use of these NPSH (GSH, NAC) can have inhibitory effect on host-derived dentinal MMPs.

4. Phytic Acid

IP6, known as inositol hexaphosphate, is the major storage form of phosphorus in plant seeds and bran (Kuwano *et al.,* 2009). IP6 level reaches up to 7% of the dry mass of some seeds (Zhou and Erdman, 1995). It is also omnipresent in mammalian cells with a concentration ranging from 10 to 100 µM (Szwergold *et al.*, 1987; Sasakawa *et al.*, 1995). IP6 in its pure form can be extracted with a low cost from rice bran (Graf, 1983).

IP6 is reported to inhibit the intestinal absorption of some minerals such as Ca^{2+}, Zn^{+2}, iron and magnesium (Hurrell, 2003). As a result of its strong negative charge (Barrientos and

Murthy, 1996), it has the ability to chelate with positively charged multivalent cations, forming complexes (Cowieson *et al.,* 2006) that are soluble under acidic conditions but precipitate at neutral pH (Schlemmer *et al.,* 2009). Recently, phytate (a salt of IP6) has been shown to have a protective role in preventing osteoporosis through decreasing the solubility of Ca salts (Lopez-Gonzalez *et al.*, 2013). This natural reagent has been reported to have a cross-linking effect on protein nano-fibers that are used for cardiac tissue engineering (Ravichandran *et al*., 2013).

Literature on the use of IP6 in dentistry is scarce; however, IP6 has been postulated to have anticariogenic or cariostatic effects through the reduction of enamel solubility (Borggreven and Driessens, 1983) or through its high affinity to hydroxyapatite, thus influencing the adsorption of bacteria to tooth surfaces [antiplaque effect] (Nordbo and Rolla, 1972). The use of IP6 instead of PA in dental silicate cement resulted in the production of acid-resistant cement through the formation of metal-phytate salts rather than an acid–base reaction and the matrix was not purely ionic as it contained a C–C linkage (Prosser *et al.,* 1983).

Based on the properties of this naturally occurring agent to chelate Ca^{+2}, form complexes with minerals and/or cross-link collagen; we hypothesized that IP6 used as an etchant/chelating agent can effectively remove the smear layer and enhance the bond strength of etch-and-rinse adhesive to dentin while having minimal effect on pulpal cells or osteoblast cells thus having the potential to replace the PA as etchant and/or EDTA as a chelating agent.

CHAPTER TWO: AIMS OF THE STUDY

These studies were conducted to evaluate the followings:

1. The effect of different concentrations of GSH on resin adhesion to PA-etched dentin.
2. The effect of different concentrations of GSH on the cytotoxicity of HEMA on pulpal-like cells.
3. The effectiveness of NPSH (GSH and NAC) in inhibiting host-derived dentinal MMPs.
4. The effect of IP6 used as etchant on the bond strength of etch-and-rinse adhesive to dentin and the topography of smear-layered dentinal surfaces.
5. The effect of IP6 on the viability of pulpal-like cells.
6. The efficacy of IP6 in removing the smear layer on NaOCl-treated flat dentin surface and instrumented root canal dentin.
7. The effect of IP6 on the viability and ALP activity of osteoblast-like cells.
8. The effect of IP6 on the bond strength of resin adhesive to NaOCl-treated dentin.
9. The reaction of IP6 to NaOCl.

CHAPTER THREE: MATERIALS AND METHODS

3.1 The Effect of Glutathione on 2-Hydroxyethylmethacrylate Cytotoxicity and on Resin-Dentin Bond Strength

Cytotoxicity test

The clonal cell line (RPC-C2A) established from dental pulp of rat incisors was used in the present study (Kasugai *et al.,* 1988). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Biowest, Instant Sterile Fetal Bovine Serum, Rue de la Caille, Nuaille, France) and antibiotic solution (60 µg/ml of kanamycin). Cultures were supplied with fresh medium every other day, and incubated in a humidified atmosphere of 95% air and 5% CO_2 and maintained at 37 ºC.

The chemicals GSH, L-Glutathione reduced, and HEMA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. Four types of solutions were prepared for cytotoxicity testing: (a) 10 mM HEMA; (b) a mixture of 10 mM HEMA and 1 mM GSH, (c) a mixture of 10 mM HEMA and 5 mM GSH and (d) a mixture of 10 mM HEMA and 10 mM GSH. To each well of 24-well culture plates, 5×10^4 cells were placed and incubated for 24 hours in a 5% CO_2 incubator at 37 ºC. Six wells were allocated for each test solution. An aliquot of 300 µL of each experimental solution was added to each well and incubated in a 5% CO_2 incubator at 37ºC for 6 hours and 24 hours. Cell culture in fresh medium without experimental solution served as the control. After the incubation times, culture medium was discarded and cells were washed with 200 µl of phosphate buffer solution to prevent any interaction between the test solutions and the colorimetric assay. A 180 µl of new culture medium was added to each well and cell viability was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics

GmbH, Germany). Twenty microliter of MTT solution was added to each well of the plate and incubated for 3 hours at 37 °C. In presence of living cells with functional mitochondria, MTT is reduced to insoluble purple formazan crystals cells. After incubation, 200 µl of dimethylsulfoxide was added to dissolve the reduced formazan crystals. The optical density (OD_{570}) of the formazan solution, which is directly proportional to the number of viable cells present in the solution, was measured with a microplate reader. A blank well was regularly used for data subtraction by placing the same volume of culture medium with MTT into culture wells. The morphology of the cultured cells was observed using phase contrast microscope (1X70, Olympus, Tokyo, Japan).

Bond strength test

Twenty extracted human non-carious third molars were used in this part of the study, according to the protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University, Japan. Flat dentin surfaces were created perpendicular to the teeth longitudinal axis, using a slow-speed diamond saw (Isomet Low Speed Saw, Buehler Ltd., Lake Bluff, IL, USA) under water lubrication. A smear layer was created on each surface using 600-grit silicon carbide paper under water irrigation. Dentin surfaces were etched using a 37% PA gel (Scotchbond Etchant, 3M ESPE St. Paul, MN, USA) for 15 seconds then rinsed for 10 seconds. The specimens were randomly divided into 4 groups according to the treatment in which a dentin surface received after etching.

In group I, dentin surfaces received no special treatment after etching (control group). In groups II, III and IV, dentin surfaces were treated with freshly prepared 2%, 5% and 10% GSH solution (L-Glutathione reduced), respectively, for 60 seconds. After dentin treatment, the surfaces were blot-dried. An etch-and-rinse adhesive (Adper Single Bond Plus, 3M ESPE) was

applied according to the manufacturer's instructions and light-cured. Resin composite (Clearfil AP-X, Kuraray Medical Inc., Tokyo, Japan) was placed over the bonded surfaces incrementally up to 5 mm thick, and light cured for 20 seconds/increment (Optilux 501, Kerr Corp., Orange, CA, USA).

The bonded specimens were stored in distilled water at 37 ºC for 24 hours. Each specimen was then sectioned perpendicular to the bonded interface, using a slow-speed diamond saw under water irrigation, into serial slabs. The latter were further sectioned to obtain (0.85 mm X 0.85 mm) composite-dentin beams. Each beam was individually fixed to a testing jig with cyanoacrylate glue and subjected to tensile loading at a cross-head speed of 1 mm/minute until failure (EZ Test, Shimadzu Crop., Kyoto, Japan). For failure analysis, the debonded specimens were air-dried, sputter-coated with gold/palladium and examined under a scanning electron microscope (SEM, JSM-5310LV scanning microscope, JEOL Ltd. Tokyo, Japan) operating at 5 kV.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 16.0 (SPSS Inc, Chicago, IL, USA). As the data were found to be normally distributed and homoscedastic, the cytotoxicity and μTBS results were separately analyzed using one-way analysis of variance (ANOVA), followed by post-hoc Tukey multiple comparison tests, at $\alpha = 0.05$.

3.2 The Inhibition Effect of Non-Protein Thiols on Dentinal Matrix Metalloproteinase Activity and HEMA Cytotoxicity

Zymographic analysis

Healthy non-carious human molar teeth were used for this part of the study, according to the protocol approved by the ethics committee. Teeth were cleaned with distilled water and any attached soft tissue was carefully removed with a scalpel. Enamel and cementum were removed with diamond burs under water irrigation and pulp tissue was carefully excavated. Fine dentin powder was prepared by pulverizing the liquid nitrogen-frozen dentin with a steel hammer (Mazzoni *et al.,* 2007). Five 1gram of aliquots of dentin powder were assigned to one of the following treatment groups: Group 1: dentin powder was partially demineralized with 1% PA for 10 minutes at 4 °C; Group 2, 3 and 4: dentin powder was partially demineralized with 1% PA for 10 minutes at 4 °C then treated, respectively, with 2% GSH (L-Glutathione reduced, Sigma Aldrich Co., St. Louis, MO), 2% NAC (Sigma Aldrich Co., St. Louis, MO) or 2% CHX (Chlorhexidine Digluconate, Wako Pure Chemical Industries Ltd., Osaka, Japan) solution for 1 minute and Group 5: untreated dentin powder that served as a control. Protein extraction was performed according to the protocol described by Breschi *et al.* (Breschi *et al.,* 2010). After treatment, all specimens were rinsed 5 times with distilled water and then resuspended for 24 hours in extraction buffer: 50 mM Tris-HCL pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3 and EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Supernatants were collected after centrifuging and protein content was precipitated with 25% trichloroacetic acid and resolubilized in loading buffer: Trizma, sodium dodecyl sulfate in water; pH 8.8. In addition, purified MMP-2 and MMP-9 (MMP Marker, Primary Cell, Ishikari, Japan) were included as

controls. Proteins were subjected to electrophoresis to assess the gelatinolytic activity under non-reducing conditions on SDS-polyacrylamide gel (NOVEX® 10% Zymogram [Gelatin] Gel, Life Technologies, CA). Activation of gelatinase proforms were achieved with 2 mM p-aminophenylmercuric acetate (APMA) at 37 °C for 1 hour (Breschi *et al.,* 2010). The SDS-polyacrylamide gel was then incubated at 37 °C for 24 hours in zymography buffer followed by staining with Coomassie Brilliant Blue R-250 and then processed in destaining buffer (Mannello *et al.,* 2003). Control zymogram was incubated in the presence of 5 mM EDTA or 2 mM 1,10-phenanthroline to inhibit gelatinases.

Cytotoxicity test

The clonal cell line (RPC-C2A) established from dental pulp of rat incisors was used in the present study (Kasugai *et al.,* 1998). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Biowest, Instant Sterile Fetal Bovine Serum, Rue de la Caille, Nuaille, France) and antibiotic solution (60 µg/ml of kanamycin). Cultures were supplied with fresh medium every other day, and incubated in a humidified atmosphere of 95% air, 5% CO_2 and maintained at 37 ºC.

Seven types of solutions were prepared for cytotoxicity testing: (a) 10 mM HEMA (Wako Pure Chemical Industries Ltd., Osaka, Japan); (b) 10 mM GSH; (c) 10 mM NAC; (d) 10 mM CHX; (e) a mixture of 10 mM GSH and 10 mM HEMA; (f) a mixture of 10 mM NAC and 10 mM HEMA and (g) a mixture of 10 mM CHX and 10 mM HEMA. To each well of 24-well culture plates, 5×10^4 cells were placed and incubated for 24 h in a 5% CO_2 incubator at 37 ºC. Six wells were allocated for each test solution. An aliquot of 300 µL of each experimental solution was added to each well and incubated in a 5% CO_2 incubator at 37ºC for 6 hours and 24 hours. Cell culture in fresh medium without experimental solution served as the control. After

the incubation times, cell culture medium was discarded and cells were washed with 200 µl of phosphate buffer solution to avoid any interaction between the experimental solutions and the colorimetric assay. A 180 µl of new culture medium was added to each well and cell viability was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH, Germany). Twenty microliter of MTT solution was added to each well of the plate and incubated for 3 hours at 37 °C. In presence of living cells with functional mitochondria, MTT is reduced to insoluble purple formazan crystals. After the incubation, 200 µl of dimethylsulfoxide was added to dissolve the reduced formazan crystals. The optical density (OD_{570}) of the formazan solution, which is directly proportional to the number of viable cells present in the solution, was measured with a microplate reader. A blank well was regularly used for data subtraction by placing the same volume of culture medium with MTT into culture wells. The morphology of the cultured cells was observed using phase contrast microscope (1X70, Olympus, Tokyo, Japan).

3.3 The Effect of Phytic Acid Used as Etchant on the Bond Strength, Smear Layer and Pulpal Cells

Bond strength test

Twenty extracted human non-carious third molars were used in this part of the study, according to the protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University, Japan. Flat dentin surfaces were created perpendicular to the tooth's longitudinal axis, using a slow-speed diamond saw (Isomet Low Speed Saw, Buehler Ltd., Lake Bluff, IL, USA) under water lubrication. A smear layer was created on each surface using 600-grit silicon carbide paper under water irrigation. The specimens were randomly divided into 4 groups according to

the type of etchant that was used to condition the dentinal surface and time of application of this etchant:

In group I, dentin surfaces were etched using 37% PA (pH 0.6) (Scotchbond Etchant, 3M ESPE St. Paul, MN, USA) for 15 seconds then rinsed with water for 10 seconds (control group). In groups II, III and IV, dentin surfaces were treated with freshly prepared 1% IP6 solution (pH 1.2) (Wako Pure Chemical Industries, Osaka, Japan) for 1 minute, 30 seconds and 15 seconds, respectively, then rinsed with water for 10 seconds. After dentin treatment, the surfaces were blot-dried and an etch-and-rinse adhesive (Adper Single Bond Plus, 3M ESPE) was applied according to the manufacturer's instructions and light-cured. Resin composite (Filtek^{TM} Z100, 3M ESPE) was placed over the bonded surfaces incrementally up to 5 mm thick, and each increment was light cured (Optilux 501, Kerr Corp., Orange, CA, USA).

The bonded specimens were stored in distilled water at 37 ºC for 24 hours. Each specimen was then sectioned perpendicular to the bonded interface, using a slow-speed diamond saw under water irrigation, into serial slabs. The latter were further sectioned to obtain (0.85 mm X 0.85 mm) composite-dentin beams. Each beam was individually fixed to a testing jig with cyanoacrylate glue and subjected to tensile loading at a cross-head speed of 1 mm/minute until failure (EZ Test, Shimadzu Crop., Kyoto, Japan). For failure analysis, the debonded specimens were air-dried, sputter-coated with gold/palladium and examined under a scanning electron microscope (SEM, JSM-5310LV scanning microscope, JEOL Ltd. Tokyo, Japan) operating at 5 kV.

SEM observation of dentin surfaces treated with PA and IP6

Flat dentin discs of 1-mm thickness were prepared from extracted human molar teeth using a low-speed diamond saw. Dentin discs were etched according to the protocol mentioned in the

bond strength test. After rinsing off the etchant for 10 seconds, specimens were dehydrated with ascending concentration of ethanol (25%, 50% and 75% for 20 minutes, 95% for 30 minutes and 100% for 60 minutes), after which, specimens were dried by immersing in hexmethyldisilazane (HMDS, Wako Pure Chemical Industries, Osaka, Japan). Specimens were dried overnight inside a covered glass vial and then sputter-coated with gold/palladium and observed under SEM operating at 5 kV.

Effect on pulpal cells viability

The colonal cell line (RPC-C2A) established from dental pulp of rat incisors was used in the present study. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Biowest, Instant Sterile Fetal Bovine Serum, Rue de la Caille, Nuaille, France) and antibiotic solution (60 µg/ml of kanamycin). Cultures were supplied with fresh medium every other day, and incubated in a humidified atmosphere of 95% air, 5% CO_2 and maintained at 37 ºC.

Four types of solutions were prepared for this test: (a) 3.7% PA [pH 1.3] (Wako Pure Chemicals); (b) 0.37% PA [pH 1.7]; (c) 0.1% IP6 [pH 2.1] and (d) 0.01% IP6 [pH 4.0]. To each well of 24-well culture plates, 5×10^4 cells were placed and incubated for 24 hours in a 5% CO_2 incubator at 37 ºC. Five wells were allocated for each test solution. An aliquot of 300 µL of each experimental solution was added to each well and incubated in a 5% CO_2 incubator at 37ºC for 6 hours and 24 hours. Cell culture in fresh medium without experimental solution served as the control. After the incubation times with the tested solutions, cell viability was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH, Germany). Thirty microliter of MTT solution was added to each well of the plate and incubated for 3 hours at 37 °C. In presence of living cells with functional mitochondria,

MTT is reduced to insoluble purple formazan crystals cells. After the incubation with MTT solution, 300 µl of dimethylsulfoxide was added to dissolve the reduced formazan crystals overnight. The optical density (OD_{570}) of the formazan solution, which is directly proportional to the number of viable cells present in the solution, was measured with a microplate reader. A blank well was regularly used for data subtraction by placing the same volume of culture medium with MTT into culture wells.

3.4 Phytic Acid: An Alternative Root Canal Chelating Agent

Smear layer removal effect

1. **Flat dentin surface**

The Human Research Ethics Committee of Tokyo Medical and Dental University, Japan reviewed this study and approved the use extracted human teeth under protocol number 725. Human non-carious third molars were used in this part of the study. Flat dentin surfaces of 1 mm thickness were created perpendicular to the tooth's longitudinal axis, using a slow-speed diamond saw (Isomet Low Speed Saw; Buehler, Lake Bluff, IL, USA) under water lubrication. A smear layer was created on each surface using 600-grit silicon-carbide paper under water irrigation. The control specimens received only a rinse with distilled water while the other specimens were treated with 5% NaOCl (Wako Pure Chemical Industries, Osaka, Japan) for 5 minutes followed by either 17% EDTA (Wako Pure Chemical Industries, Osaka, Japan) for 1 minute, 1% IP6 (Wako Pure Chemical Industries, Osaka, Japan) for 1 minute or 30 seconds or distilled water. All solutions were applied with agitation using a microbrush. After rinsing with distilled water for 10 seconds, specimens were dehydrated with ascending concentrations of ethanol (25, 50, and 75% for 20 minutes, 95% for 30 minutes, and 100% for 60 minutes),

followed by immersion in hexamethyldisilazane (HMDS; Wako Pure Chemical Industries). Specimens were dried overnight inside a covered glass vial and then sputter-coated with gold/palladium and observed under a scanning electron microscope (SEM) (JSM-5310LV scanning microscope; JEOL, Tokyo, Japan) operating at 5 kV.

2. Root canal surface

Single-rooted maxillary human canines with straight root and mature apex were used in this part of the study. Each tooth was decoronated at 17 mm from the anatomic apex. The experimental setup was followed according to the closed system proposed by Tay *et al.* (Tay *et al.*, 2010) in which the root apex was covered with glue to prevent fluid extrusion through the apical foramen. Protaper NiTi rotary instruments (Dentsply Maillefer; Ballaigues, Switzerland) were used to prepare and shape the canals according to the manufacturer's instructions ending with the finishing file number 3. Between each file, the canals were irrigated with 1 ml of 5% NaOCl. The canals were rinsed with distilled water before the application of the chelating agents. A final rinse of 1 ml 17% EDTA for 1 minute or 1%IP6 for 1 minute or 30 seconds was performed with the solutions gently agitated inside the canal using a hand k-file number 15. The canals were then irrigated again with distilled water and dried with absorbent paper points. The control group received no treatment after instrumentation expect for a final rinse with distilled water. To facilitate the separation of the root into two halves, deep longitudinal grooves were prepared on the external root surface without perforating the canals followed by splitting the root using a hammer and chisel. The dehydration process for SEM observation was conducted in the same previously mentioned manner. Representative images of the middle and apical thirds at a 1000x magnification were taken for each group.

Effect on cells viability and ALP activity

1. Cell viability assay

The clonal cell line (MC3T3-E1), osteoblast-like-cells, established from mouse calvaria, was used in the present study. To each well of 24-well culture plates, MC3T3-E1 cells ($5X10^4$ cells/well) were placed and incubated for 24 hours in a 5% CO_2 incubator at 37 ºC. Six wells were allocated for each test solution. The test solutions included various concentrations (500-10,000 µg/ml culture medium) of either 17% EDTA or 1%IP6. An aliquot of 300 µL of each experimental solution was added to each well and incubated in a 5% CO_2 incubator at 37 ºC for 24 hours. Cell culture in fresh medium without experimental solution served as the control. After the incubation time, culture medium was discarded and cells were washed with 200 µl of phosphate buffer solution to prevent any interaction between the test solutions and the colorimetric assay. A 180 µl of new culture medium was added to each well and cell viability was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics GmbH, Germany). Twenty microliter of MTT solution was added to each well of the plate and incubated for 3 hours at 37 °C. After adding 200 µl of dimethylsulfoxide was added to dissolve the optical density (OD_{570}) of the formazan solution, which is directly proportional to the number of viable cells present in the solution, was measured with a microplate reader. The morphology of the cultured cells was observed using phase contrast microscope (1X70, Olympus, Tokyo, Japan).

2. ALP activity measurement

Cultured MC3T3-E1 cells ($5X10^4$ cells/well) were treated with 17% EDTA or 1% IP6 at a concentration of 500 µg/ml of culture medium for 1, 7, and 14 days. Both the medium and the test solution were refreshed every 3 days. ALP activity was determined using ALP Assay Kit

(Takara Bio Inc., Shiga, Japan). After each incubation time, cell culture medium was aspirated and each well was rinsed with 300 µl of 0.9% sodium chloride. Following aspiration of sodium chloride, 100 µl of extraction solution was added to each well and cells were manually scraped. Substrate solution (100 µl) was added to each well and let react at 37 °C for 30 minutes followed by the addition of 0.5 nM sodium hydroxide (stop solution, 100µl). After adding the stop solution, absorbency at 405 nm of 100 µl of each solution was immediately measured using a plate reader.

For statistical analysis, the mean optical density of the MTT test and ALP activity of the control group at 24 hours was set to represent 100%, and results of the experimental groups were expressed as percentages of the control. Statistical analysis was performed by applying two-way analysis of variance (ANOVA) using the tested materials and concentrations or incubation periods as two factors, further by one-way analysis of variance (ANOVA). In case of significance, statistical analyses were performed by Tukey multiple comparison test (α=0.05).

Effect on resin adhesion to NaOCl-treated dentin

Twenty extracted human non-carious third molars were used in this part of the study. Flat dentin surfaces were created in the same previously described manner. A smear layer was created on each surface using 600-grit silicon-carbide paper under water irrigation. The specimens were randomly divided into five groups according to the type of treatment each dentin surface received. Dentin surfaces were conditioned in the same way described earlier for the effect on smear layer removal on flat surface. After treatment, a final rinse with distilled water was performed then dentin surfaces were blot-dried and one-step self-etch adhesive (Scotchbond™ Universal Adhesive; 3M ESPE, St Paul, MN, USA) was applied, according to the manufacturer's

instructions, and light cured. Resin composite (Filtek Z100; 3M ESPE) was placed over the bonded surfaces incrementally up to a thickness of 5 mm, and each increment was light cured (Optilux 501; Kerr, Orange, CA, USA). The bonded specimens were stored in distilled water at 37 °C for 24 hours. Each specimen was sectioned to obtain composite–dentin beams of 0.85 mm x 0.85 mm. Each beam was individually fixed to a testing jig with cyanoacrylate glue and subjected to tensile loading at a cross-head speed of 1 mm/minute. For failure analysis, the debonded dentin specimens and examined under a SEM operating at 5 kV.

Reaction between the used reagents

One ml of 5% NaOCl was mixed with either 1 ml of 17% EDTA or 1% IP6 and changes in temperature, pH and bubble formation were recorded. After 1 minute of mixing, 200 µl of each mixture was added on a hematotropin-stained paper to observe the bleaching effect and compare it to the bleaching effect of pure 5% NaOCl.

CHAPTER FOUR: RESULTS

4.1 The Effect of Glutathione on 2-Hydroxyethylmethacrylate Cytotoxicity and on Resin-Dentin Bond Strength

Effect of tested materials on cell viability

The results of the cytotoxicity tests (6 hours and 24 hours) are presented in Figure 1. For both exposure times, 10 mM HEMA group and 10 mM HEMA+1mM GSH group had an OD values that were significantly lower from the control ($P < 0.001$). At 6-hour exposure time 10 mM HEMA+ 5 mM GSH group did not show a statistical significant difference from the control ($P = 0.57$) while at 24 hours it had a significantly lower OD value when compared to the control ($P < 0.001$). At the 6-hour exposure time, 10 mM HEMA+ 10 mM GSH group showed an OD value that was not statistically significant from the control ($P = 0.99$), while at 24 hours it had a significantly higher OD value when compared to the control ($P < 0.001$).

Figure 2 shows the morphology of the cells after 24 hours of incubation in tested solutions. Morphologically, the cultured RPC-C2A cells were polygonal-shaped and showed fibroblast-like characteristic (Figure 2a). Cells treated with 10 mM HEMA became rounded with cellular retraction, and increase in intercellular spaces was observed (Figure 2b). For 10 mM HEMA+ 1 mM GSH group, cells lost their normal morphology and became smaller and rounded at both 6-hour (Data not shown) and 24-hour (Figure 2c) exposure times. At 6-hour exposure time 10 mM HEMA+ 5 mM GSH retained the normal morphology of the cells (Data not shown) while at 24 hours cellular shrinkage was noticed with increased intercellular spaces (Figure 2d). At both exposure times of the 10 mM HEMA+10 mM GSH cells retained their normal morphology (Figure 2e).

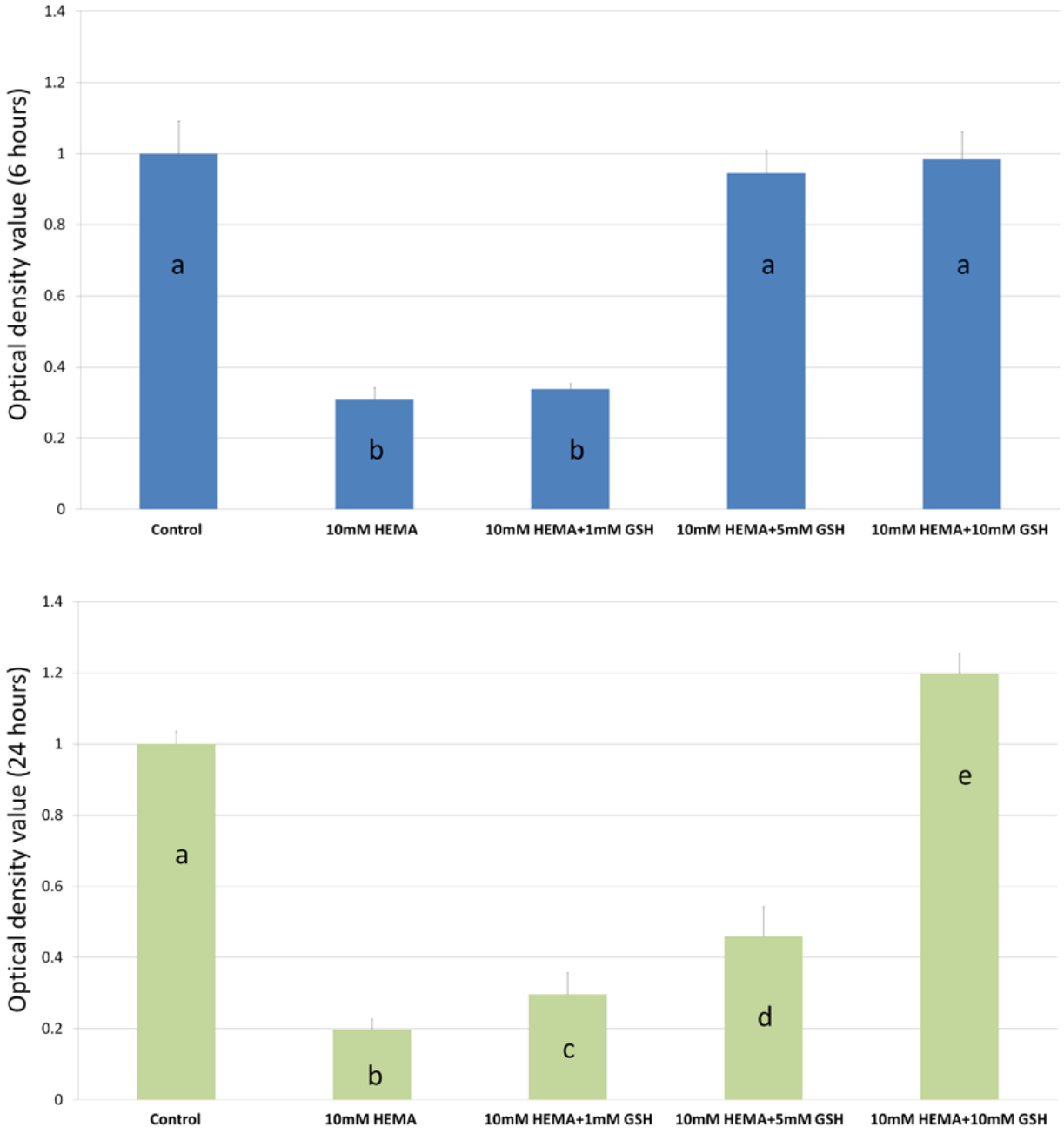

Figure 1 Cytotoxicity of the tested solutions with culture medium on rat dental pulp cells (6 hours and 24 hours). MTT results of tested solutions (n=6), the average of each group was transformed into ratio which represents the percentage of mitochondrial activity of the cells in comparison to the control group which was defined as having 100% mitochondrial activity inside the cells. Same letter represents no significant difference ($P > 0.05$).

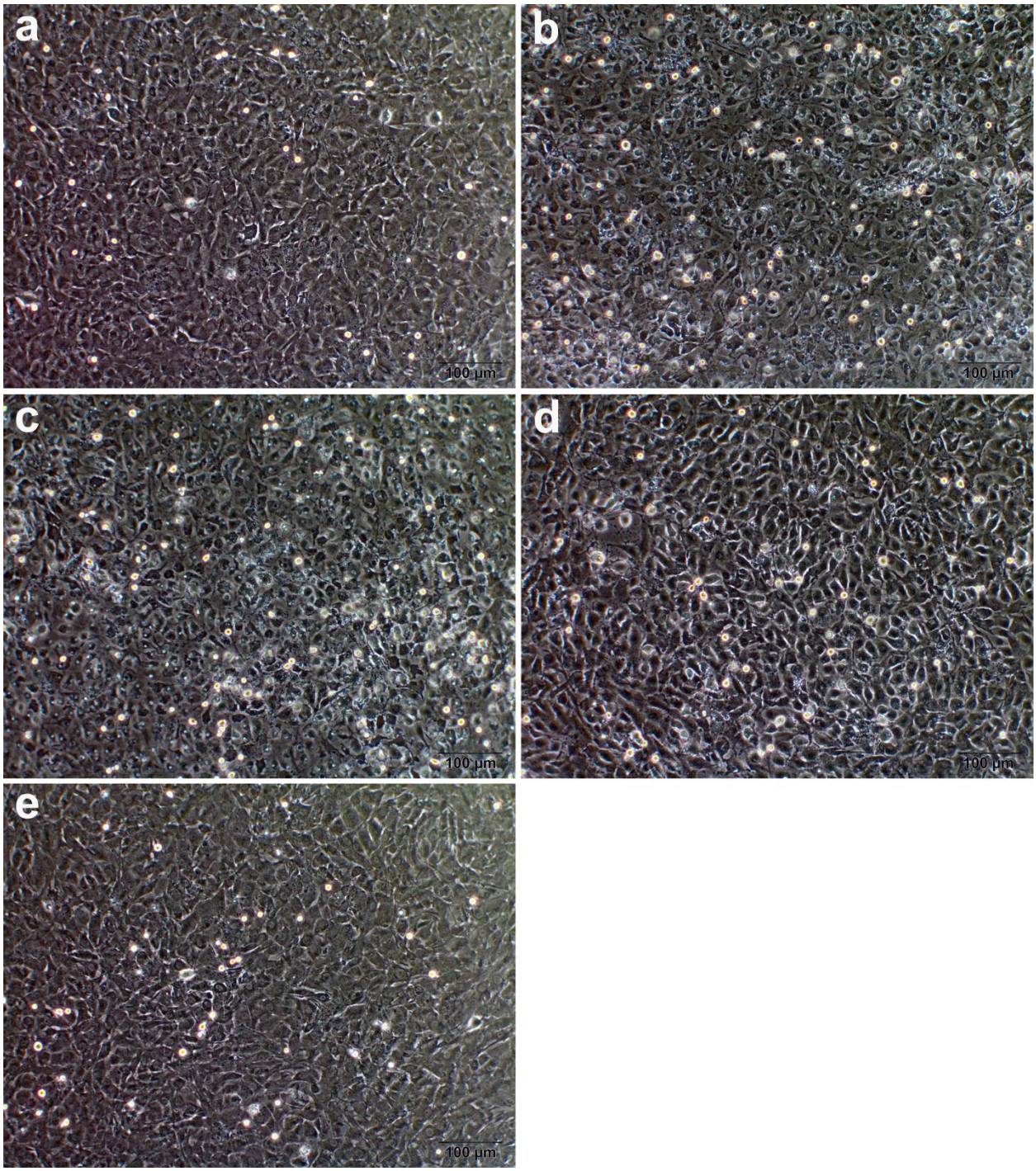

Figure 2 (a-e) Morphological changes of pulpal cells after 24-hour exposure time to the tested solutions. (a) Control pulp cells: polygonal-shaped cells. (b) HEMA-treated cells and (c) 10 mM HEMA+1 mM GSH-treated cells: decreased density of the cells and marked observation of cell degeneration. (d) 10 mM HEMA+5 mM GSH-treated cells: cells appeared smaller and increased intercellular spaces can be observed. (f) 10 mM HEMA+10 mM GSH-treated cells: no morphological changes when compared to the control group, with the cells being normal in size and polygonal-shaped.

µTBS and failure analysis

Means and standard deviations of the µTBS values of the groups are presented in Table 1. Significant difference was observed among the tested groups ($P < 0.05$). The Tukey test further indicated that the µTBS of 2% [40.8 ± 12.4 MPa], 5% [43.2 ± 13.0 MPa] and 10% [36.6 ± 11.3 MPa] GSH-treated group was not significantly different from the control group [40.6 ± 14.3 MPa] ($P > 0.05$). There was no significant difference ($P > 0.05$) between 2% GSH-treated group and 5% GSH-treated group; however, 5% GSH-treated group showed a statistically higher µTBS when compared to 10% GSH-treated group ($P = 0.003$). Scanning electron microscope observations indicated that the fracture pattern of all groups was mostly cohesive failure at the bottom of the hybrid layer. Dentinal tubules were exposed, showing fractured resin tags inside the tubules (Data not shown).

Table 1 Mean microtensile bond strength values (MPa ±SD) of experimental groups

Group	*n*	Mean (Mpa) ± SD
Group I (Control)	75	40.6 ± 14.3^{ab}
Group II (2% GSH-pretreatment)	74	40.8 ± 12.4^{ab}
Group III (5% GSH-pretreatment)	87	43.2 ± 13.0^{a}
Group IV (10% GSH-pretreatment)	88	36.6 ± 11.3^{b}

Groups identified by different superscript letters indicate significant statistical difference (one-way ANOVA with Tukey post hoc test, $\alpha = 0.05$). SD, standard deviation. There was no significant difference between each test group and the control ($P > 0.05$). However, group III showed a statistically higher microtensile bond strength when compared to group IV ($P = 0.003$).

4.2 The Inhibition Effect of Non-Protein Thiols on Dentinal Matrix Metalloproteinase Activity and HEMA Cytotoxicity

Zymographic analysis

Areas of proteolytic activity in zymograms appear as clear bands against the darkly stained background of the undigested substrate in the gel (Figure 1). Zymograms of mineralized dentin showed faint proteolytic activity that is related to the active form of MMP-2 (62-kDa) while, PA-demineralized dentin showed clearer bands of MMP-2 pro- and active forms (72-kDa and 62-kDa, respectively). Treatment with 2% GSH, 2% NAC or 2% CHX resulted in decreased gelatinolytic activities of pro- and active forms of MMP-2.

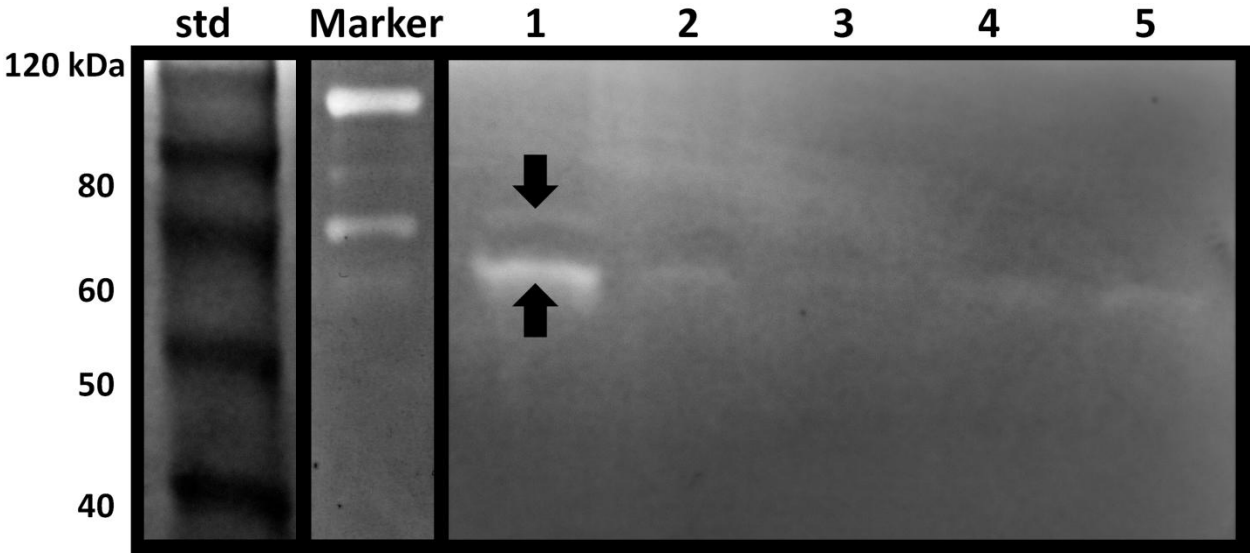

Figure 1 Results of zymographic analysis, **Lane 1:** Phosphoric acid-demineralized dentin powder extract showed gelationlytic activity of pro-MMP-2 (72 kDa, upper pointer) and active MMP-2 (62 kDa, lower pointer). **Lane 2, Lane 3 and Lane 4** "respectively"**:** Activity of extract from GSH-, NAC- and CHX-treated demineralized dentin powder resulted in decreased gelatinolytic activities of pro- and active forms of MMP-2. **Lane 5:** Activity of extract from untreated mineralized dentin powder showed faint activity of active MMP-2 while no pro-MMP2 activity was detected. The marker lane is purified MMP-2 and MMP-9 proteins that were used as controls.

Cytotoxicity test

The results of the cytotoxicity tests (6 hours and 24 hours) are presented in Figure 2. For both exposure times, HEMA, CHX and CHX+HEMA groups showed significantly lower OD values when compared with the control ($P < 0.001$).

At 6-hour exposure time GSH, NAC, GSH+HEMA and NAC+HEMA groups did not show statistically significant difference when compared to the control ($P > 0.05$); however at the 24-hour exposure time they had a statistically significantly higher OD values when compared with the control ($P \leq 0.001$). There was no significant difference among GSH, NAC, GSH+HEMA and NAC+HEMA groups at the 6-hour and 24-hour exposure times ($P > 0.05$).

Morphologically, the cultured RPC-C2A cells were polygonal-shaped and showed fibroblast-like characteristic (Figure 3a). Cells treated with HEMA for 6 hours became rounded with cellular retraction and increases in intercellular spaces (Figure 3b). For the CHX containing groups, cells morphology was disrupted and cellular debris could be detected (Figure 3e,h). For all groups containing GSH and NAC the cells retained their normal morphology (Figure 3c,d,f,g).

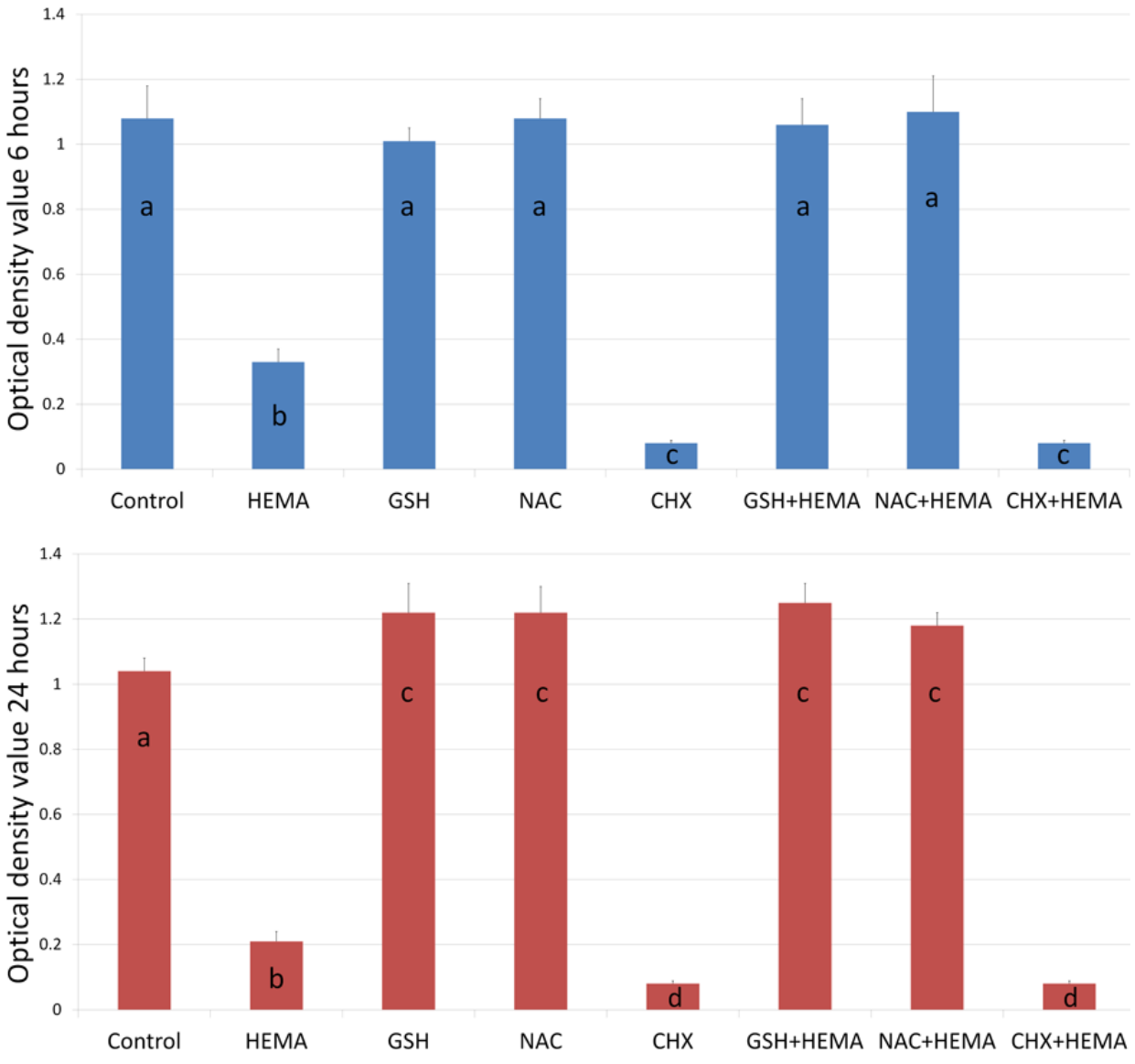

Figure 2 Cytotoxicity of the tested solutions with culture medium on rat dental pulp cells (6 hours and 24 hours; respectively). MTT results of tested solutions (n=6). Same letter represents no significant difference ($p > 0.05$).

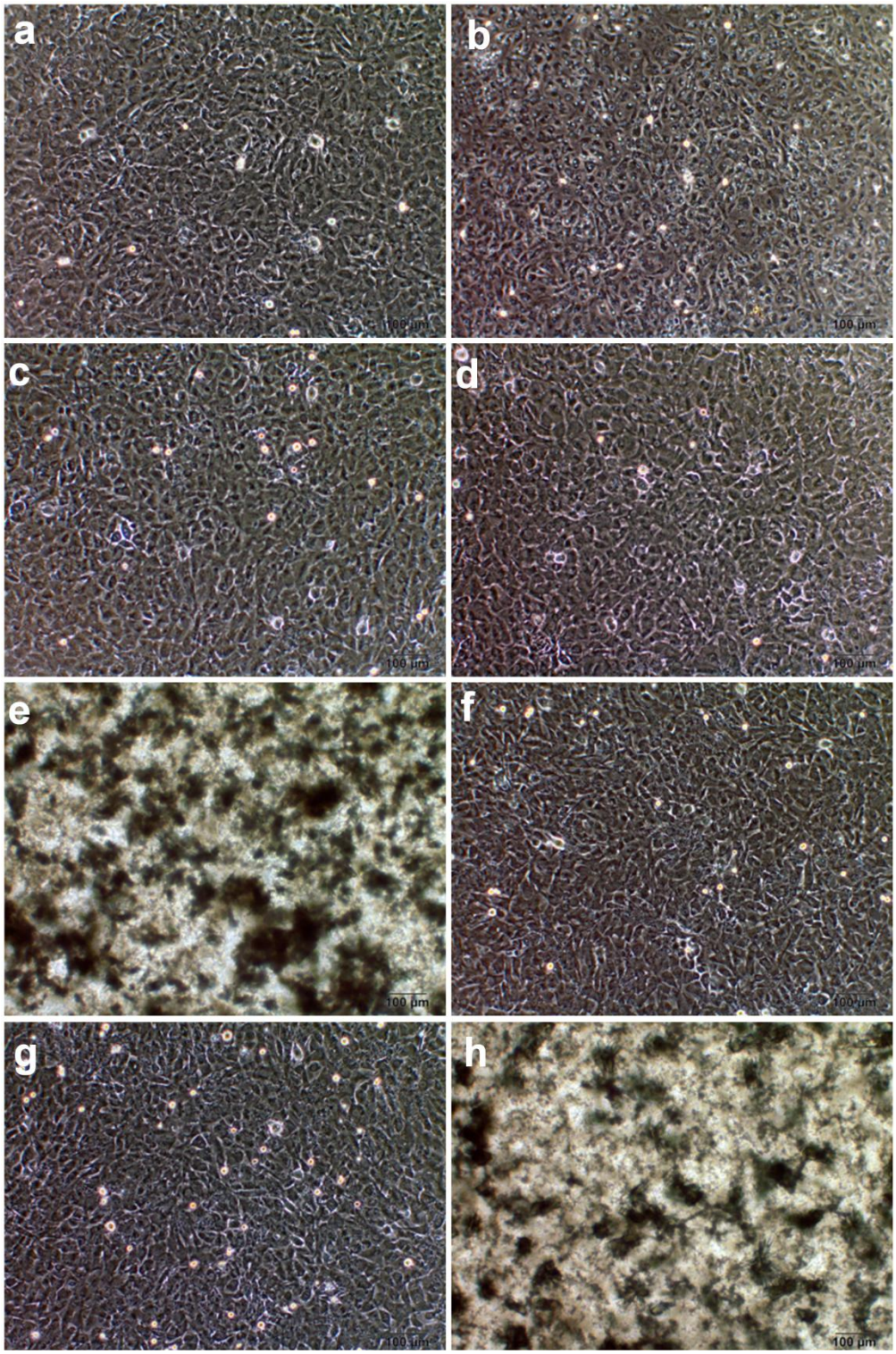

Figure 3 Morphological changes of pulp cells after 6 hours of exposure to the tested solutions. (a) Control pulp cells: polygonal-shaped cells. (b) HEMA-treated cells: decreased density of the cells and reduced cells size. (c,d) GSH- and NAC-treated cells, respectively: cells appeared normal, with no morphological changes when compared with the control group. (e) CHX-treated cells: marked disruption of cells morphology. (f,g) GSH+HEMA- and NAC+HEMA-treated groups, respectively: no morphological changes when compared with the control group. Cells are normal in size and polygonal-shaped. (h) CHX+HEMA-treated group: the images detected were similar to CHX-treated group.

4.3 The Effect of Phytic Acid Used as Etchant on the Bond Strength, Smear Layer and Pulpal Cells

Bond strength test

Means and standard deviations of the μTBS values of the groups are presented in Table 1. Significant difference was observed among the tested groups ($P < 0.05$). The Tukey test further indicated that the μTBS of 1% IP6 applied for 1 minute [72.5 ± 19.1 MPa], 30 seconds [70.0 ± 23.4 MPa] and 15 seconds [66.0 ± 20.9 MPa] was significantly different from the control group [49.3 ± 14.9 MPa] ($P < 0.05$). There was no significant difference ($P = 0.225$) between 1% IP6 applied for 1 minute, 30 seconds or 15 seconds. Figure 1 shows representative samples for the fracture mode of each group. Scanning electron microscope observations indicated that the fracture pattern of groups treated with IP6 was predominately mixed failure that involved the adhesive and the hybrid layer, whereas for the control group the fracture was mostly cohesive failure in the hybrid layer.

Table 1 Mean microtensile bond strength (μTBS) values (MPa ±SD) of experimental groups

Group	*n*	Mean (Mpa) ± SD
Group I (Control)	68	49.3 ± 14.9^{a}
Group II (1% IP6/1 minute)	67	72.5 ± 19.1^{b}
Group III (1% IP6/ 30 seconds)	67	70.0 ± 23.4^{b}
Group IV (1% IP6/ 15 seconds)	65	66.0 ± 20.9^{b}

Groups identified by different superscript letters indicate significant statistical difference (one-way ANOVA with Tukey post hoc test, alpha = 0.05). SD, standard deviation.

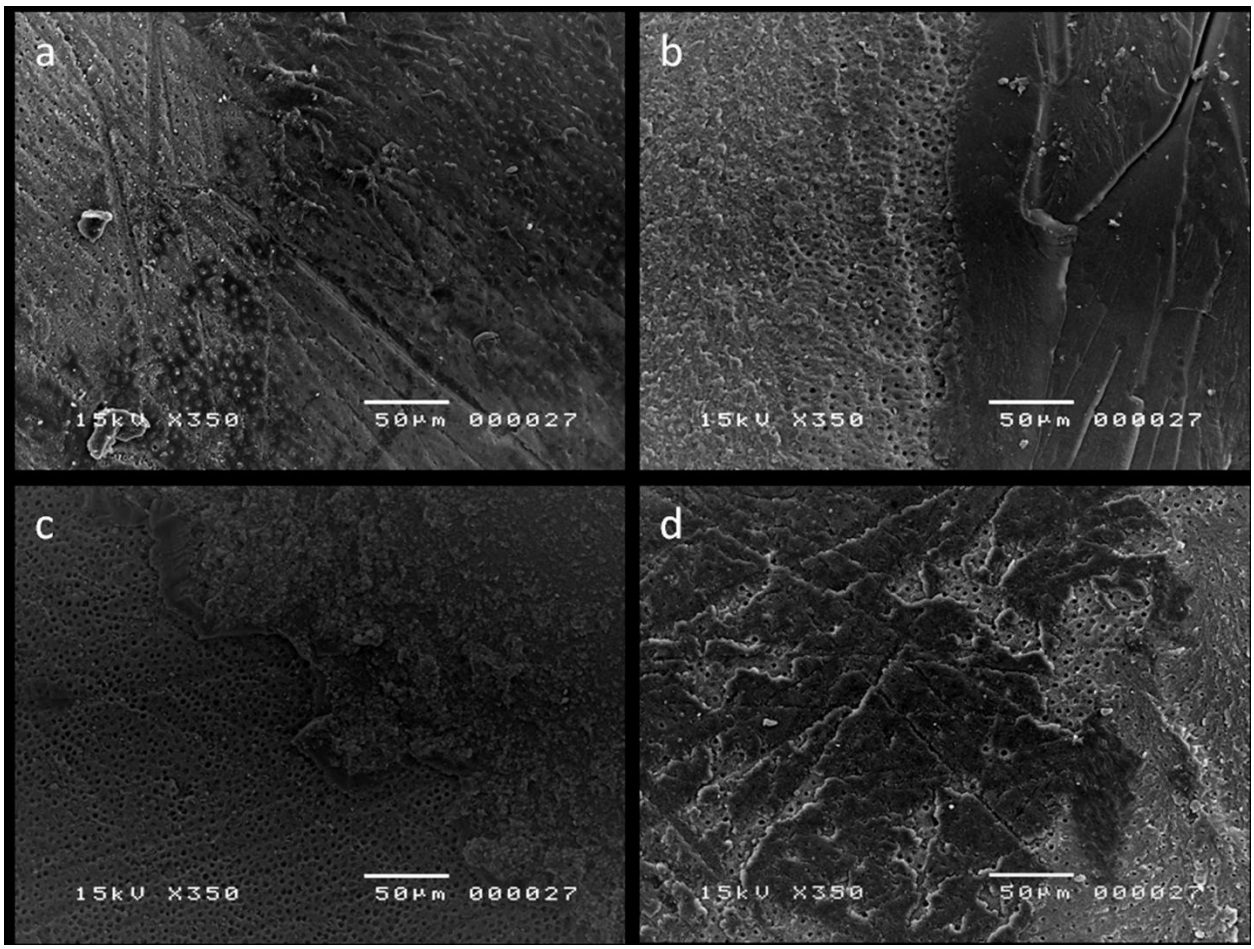

Figure 1 Scanning electron microscopy images of the dentin side of the fractured specimens. (a) Control group: the main scheme of the fracture was cohesive in the hybrid layer, (b, c,d) 1% IP6 [1 minute, 30 seconds and 15 seconds; respectively]: these fractures were mostly mixed where they involved the adhesive and the hybrid layer.

SEM observation of dentin surfaces treated with PA and IP6

Figure 2 shows the topographical changes on smear-layered dentin surfaces after etching with 37% PA or 1% IP6. The 600 silicon carbide paper produced uniform scratches with smear layer covering the surface (Figure 2a). The application of 37% PA for 15 seconds resulted in removal of the smear layer and tubular plugs. The tubular orifices were open and clean, and there was almost no intratubular dentin (Figure 2b). IP6 used for 1 minute, 30 seconds or 15 seconds was

effective to remove the smear layer, leaving a clean surface, open dentinal tubules and an exposed collagen network (Figure c-e) that can be more clearly observed than the group treated with PA.

Effect on pulpal cells viability

The results of the effect of PA and IP6 (6 hours and 24 hours) on pulpal cells are presented in Figure 3. Both PA concentrations showed significantly lower OD values when compared with the control at both exposure times ($P < 0.001$). There was no significant difference between the OD value obtained with 3.7% PA and the one obtained with 0.37% PA at both exposure times ($P > 0.05$).

At 6-hour exposure time, 0.1% IP6 showed a significantly higher OD value when compared to the control ($P < 0.001$) while at 24-hour exposure time the OD value of 0.1% IP6 was significantly lower than the control ($P = 0.029$). The OD value obtained with 0.01% IP6 at 6-hour exposure time was significantly higher than the control ($P = 0.003$), whereas there was no statistical significance at 24-hour exposure time ($P = 0.103$). The OD value was significantly higher at 6-hour exposure time with 0.1% IP6 when compared to 0.01% IP6 ($P < 0.001$); however, at 24-hour exposure time 0.01% IP6 obtained a significantly higher OD value than 0.1% IP6 ($P < 0.001$). At both exposure times 0.1% and 0.01% IP6 showed a significantly higher OD values when compared to 3.7% and 0.37% PA ($P < 0.001$).

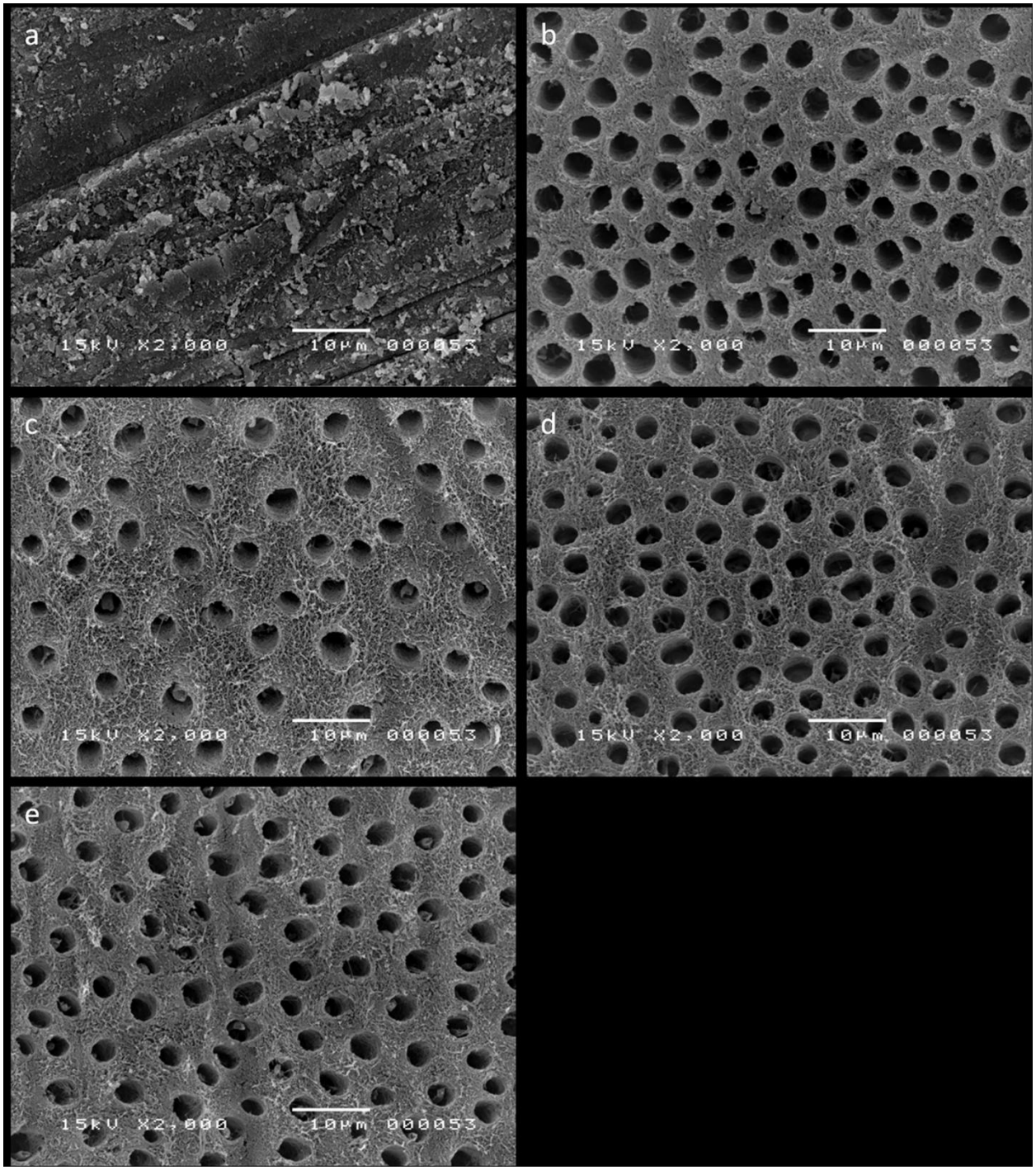

Figure 2 (a) Representative scanning electron microscopy image of the smear layer produced by 600-grit silicon carbide paper. (b-e) Representative images for the effect of the used etchants on the smear layer produced by 600-grit silicon carbide paper; (b) PA-etched dentin: complete removal of the smear layer and plugs, with open dentinal tubules, (c-e) IP6-etched dentin for 1 minute, 30 seconds and 15 seconds, respectively: dentinal surfaces are clean and free of smear layer and plugs, with open dentinal tubules. Collagen network can be recognized more easily at this magnification level than for PA-etched dentin.

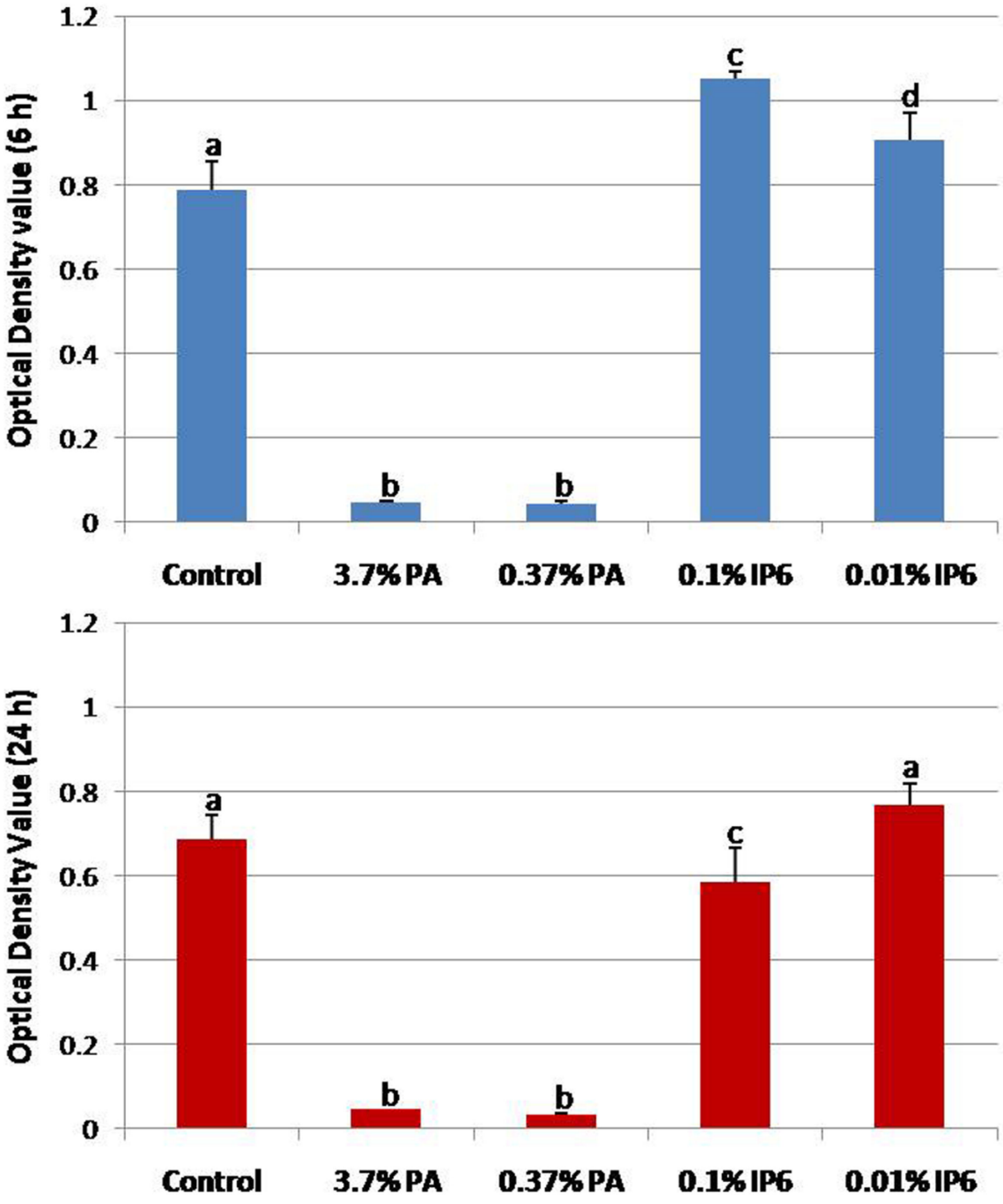

Figure 3 Cytotoxicity of the tested solutions with culture medium on rat dental pulp cells at 6 hours (upper) and 24 hours (lower). MTT results of tested solutions (n=5). Same letter represents no significant difference ($P > 0.05$).

4.4 Phytic Acid: An Alternative Root Canal Chelating Agent

Smear layer removal effect

1. Flat dentin surface

Figure 1 shows the topographical changes of smear layered-flat dentin surfaces conditioned with 17% EDTA for 1 minute or 1% IP6 for 1 minute or 30 seconds after an initial treatment with 5% NaOCl for 5 minutes. The 600-grit silicon-carbide paper produced uniform smear layer covering the whole surface (Figure 1A). The 5-minute application of 5% NaOCl resulted in partial removal of the smear layer (Figure 1B).

The use of 17% of EDTA for 1 minute after NaOCl resulted in almost complete removal of the smear layer (Figure 1C), which can also be seen in the case of 1% IP6 for 1 minute or 30 seconds (Figure 1D, E; respectively); however, wider openings of the dentinal tubules are observed in the case of 1% IP6 in both application times when compared to 17% EDTA.

2. Root canal surface

Figure 2 shows the effect of different irrigation regimens on the removal of the smear layer from the middle and apical thirds of instrumented root canals. A smear layer covering the entire surface was shown in canals instrumented and irrigated with 5% NaOCl and a final rinse with distilled water (Figure 2A). Figure 2B represents the effect of 17% EDTA applied for 1 minute on the middle third; a cleaner surface, few areas covered with smear layer and open dentinal tubules were observed, while it showed less effectiveness in the apical third where smear layer was still covering much part of the surface with less open dentinal tubules and some debris (Figure 2C).

The use of 1% IP6 for 1 minute or 30 seconds resulted in a clean, debris free, and open dentinal tubules in the middle third (Figure 2D,F; respectively) while the canal wall at the apical third was covered with some smear layer and debris (Figure 2E, G; respectively).

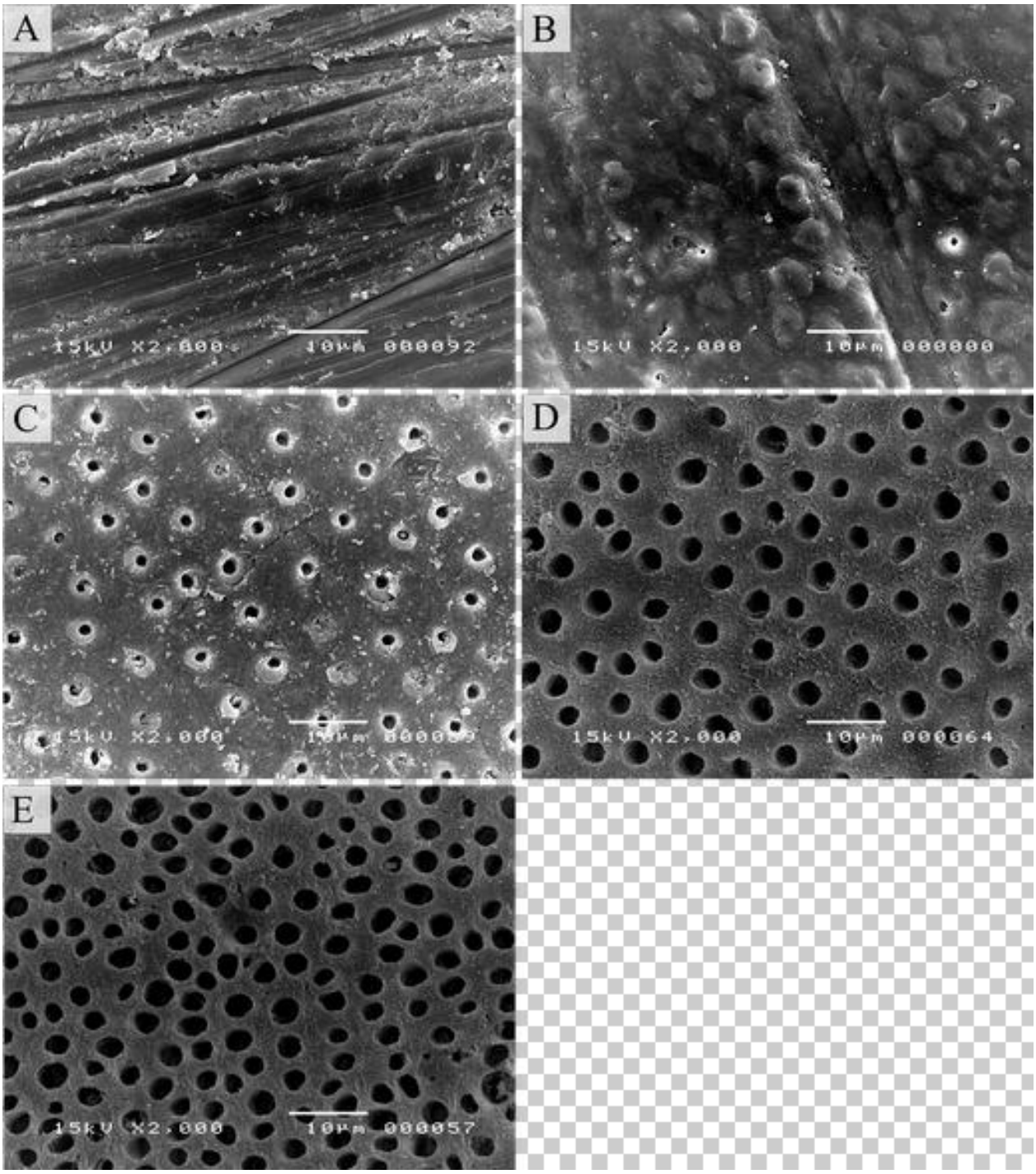

Figure 1 Representative scanning electron microscopy images of the effect of different treatment in removing the smear layer from flat coronal dentin surfaces. (A) Smear layer produced by 600 silicon carbide paper. (B) 5% NaOCl applied for 5 minutes, where the ineffectiveness of NaOCl to remove the smear layer was observed. (C) 17% EDTA applied for 1 minute on NaOCl-treated flat coronal dentin surface, which resulted in cleaner dentinal surface with open dentinal tubules. (D) and (E) 1% IP6 applied for 1 minute or 30 seconds, respectively. IP6 resulted in a clean and debris-free surface with widely open dentinal tubules.

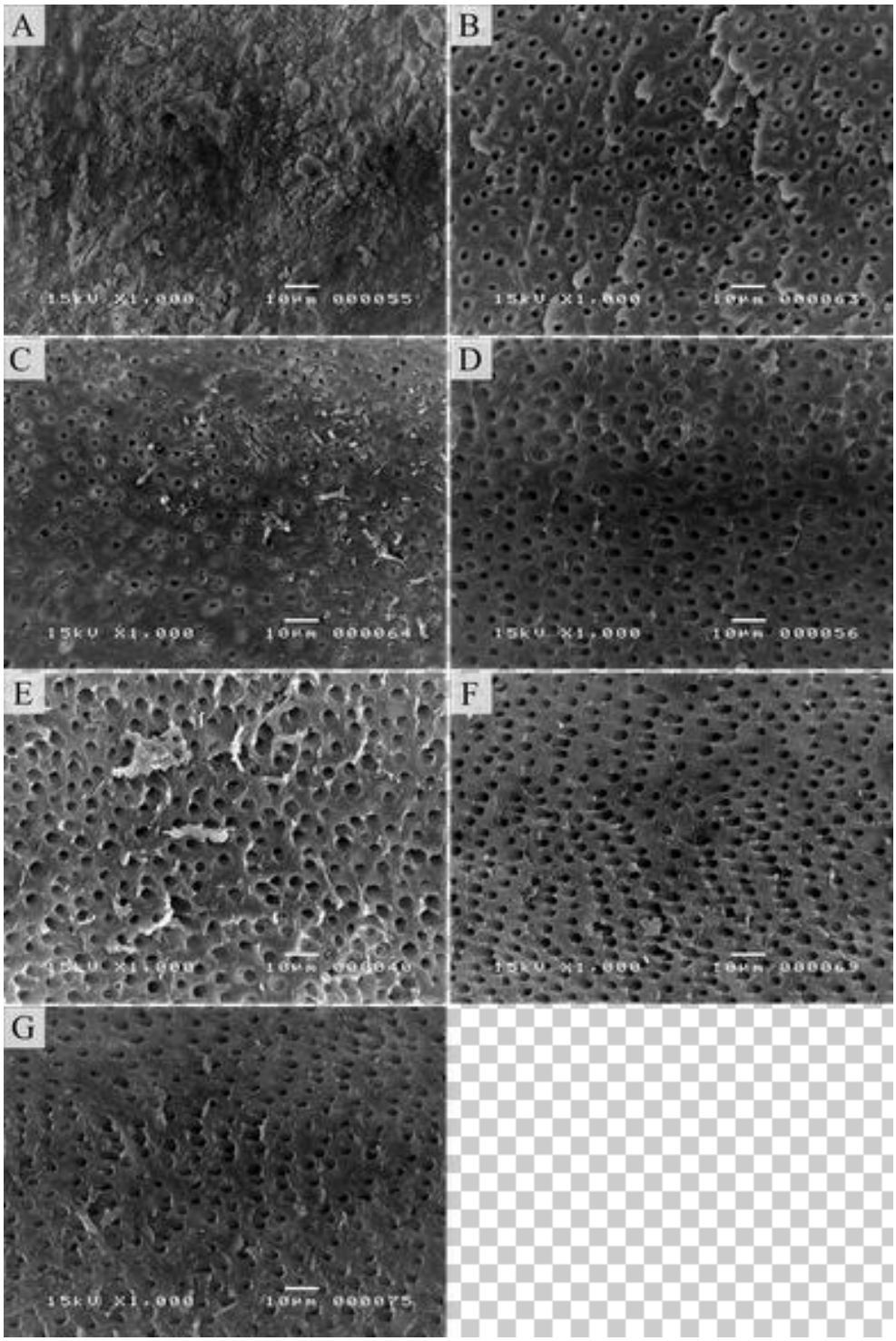

Figure 2 Representative scanning electron microscopy images of the effect of different irrigation regimens on the removal of the smear layer from the middle and apical thirds of instrumented root canals. (A) Smear layer produced on root canal surface after instrumentation and irrigation with 5% NaOCl with a final rinse of distilled water. (B) and (C) Smear layer removal from instrumented root canals that received a final flush of 17% EDTA for 1 minute, middle and apical thirds, respectively. (D) and (E) Smear layer removal from instrumented root canals that received a final flush of 1% IP6 for 1 minute, middle and apical thirds, respectively, whereas (F) and (G) received a final rinse of 1% IP6 for 30 seconds, middle and apical thirds, respectively.

Effect on cells viability and ALP activity

The effect of various concentrations of 1% IP6 and 17% EDTA on the viability of MC3T3-E1 after 24-hour of exposure is shown in Figure 3A. The data are expressed as percentage of the OD value in the control cells. Two-way ANOVA showed that the factor "material" and "concentration" were significant ($P < 0.001$) the interaction between these two factors was also significant ($P < 0.001$). EDTA negatively affected the viability of the cells in a dose dependent manner. The three highest concentrations of EDTA caused a significant decrease in cells viability, whereas the lower concentrations were biocompatible. The OD value obtained for the 5000 µg/ml [EDTA] was significantly higher than the 10.000 µg/ml and 7.500µg/ml [EDTA] ($P < 0.001$); however, it was significantly less when compared to the OD values of 2.500 µg/ml and 500 µg/ml [EDTA] ($P < 0.001$). The OD values of latter two concentrations were not significantly different ($P = 0.921$). All tested concentrations of IP6 had no negative effect on cells viability. The OD value of the 500 µg/ml [IP6] was significantly higher than those of 10.000 µg/ml ($P = 0.004$) and 7.500 µg/ml ($P = 0.009$) [IP6], however, it was not significant when compared to 5.000 µg/ml ($P = 0.145$) and 2.500 µg/ml ($P = 0.052$) [IP6].

For ALP activity assay, two-way ANOVA showed that the "material" and "incubation time" factors were significant ($P < 0.001$) but the interaction between these two factors was not significant ($P = 0.057$). The effect of the 500 µg/ml concentration of 17% EDTA or 1% IP6 on ALP activity at 1, 7 and 14 days is shown in Figure 3B. A significant decrease in ALP activity when compared to the control was noticed at 1 day ($P = 0.03$) and 14 days ($P = 0.021$) in EDTA group. In contrast, IP6 group did not affect the ALP activity when compared to the control group at the three incubation times ($P > 0.05$).

Morphologically, MC3T3-E1 cells in the control media showed the polygonal appearance (Figure 4A) while cells treated with EDTA at the two highest concentrations exhibited contracted, spherical morphology and increases in intercellular spaces which are indicators of cellular death and decreased proliferation (Figure 4B,C). For the 5,000 µg/ml of EDTA, some cells maintained the normal morphology; however, cellular density was less and intercellular spaces were increased (Figure 4D). The 2,500 µg/ml (Figure 4E) and 500 µg/ml of EDTA (data not shown) did not affect the morphology of the cells when compared to the control. For the various concentrations of IP6, cells retained their normal polygonal morphology (Figure 4F-I).

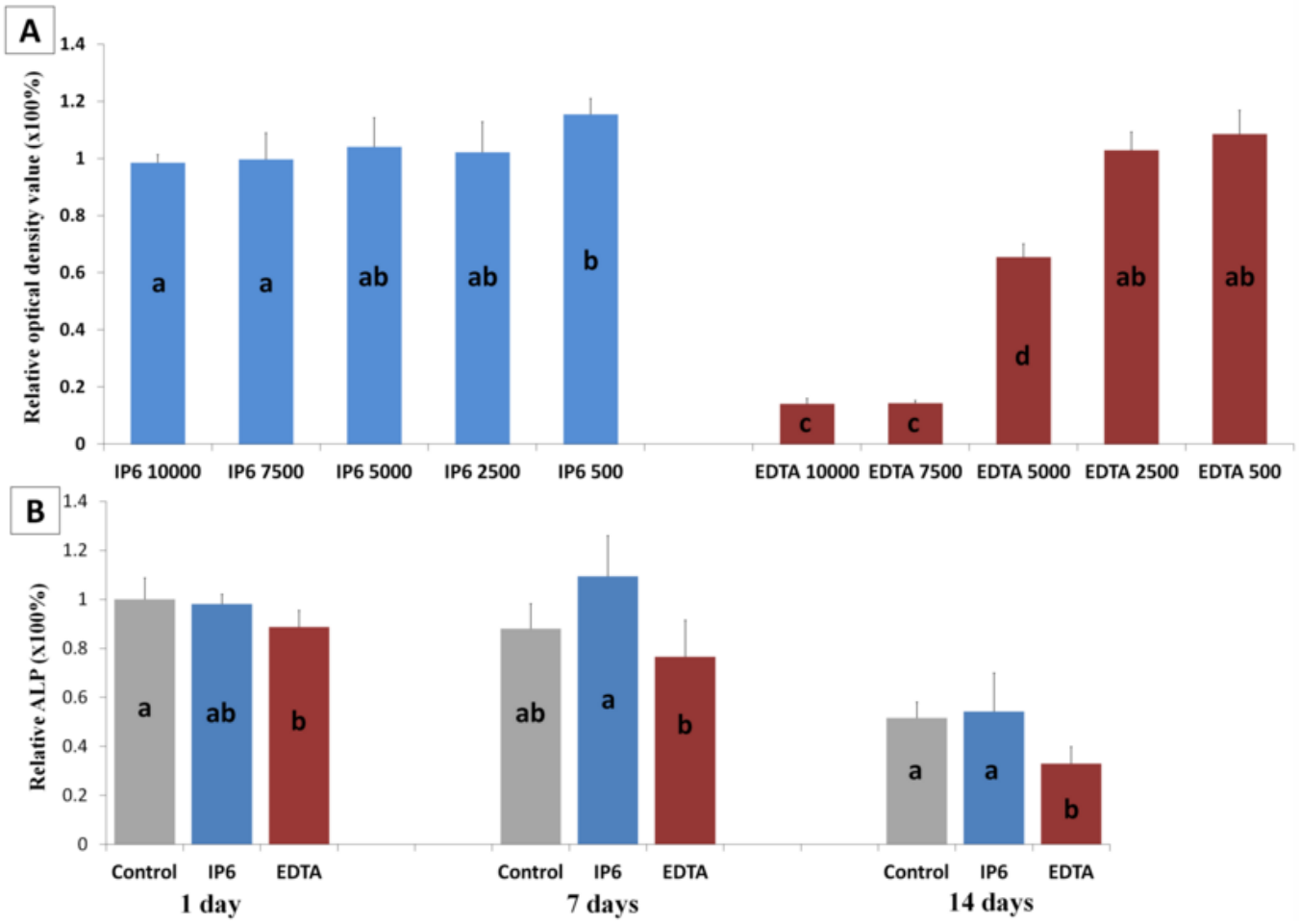

Figure 3 (A): Cytotoxicity of culture medium containing the different concentrations of the test solutions of 1% IP6 or 17% EDTA on osteoblast-like cells after 24 hours of incubation. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (n = 6 each group). Two-way ANOVA indicated an interaction between materials and concentrations. The same lowercase letter indicates no significant difference ($P > 0.05$)**. (B):** ALP activity of osteoblast-like cells cultured with 500 µg/ml 17%EDTA or 1% IP60 for 1, 7 and 14 days. Data were expressed as percentage of ALP activity in the control cells on day 1. Data were analyzed using one-way ANOVA. The same lowercase letter indicates no significant difference ($P > 0.05$)

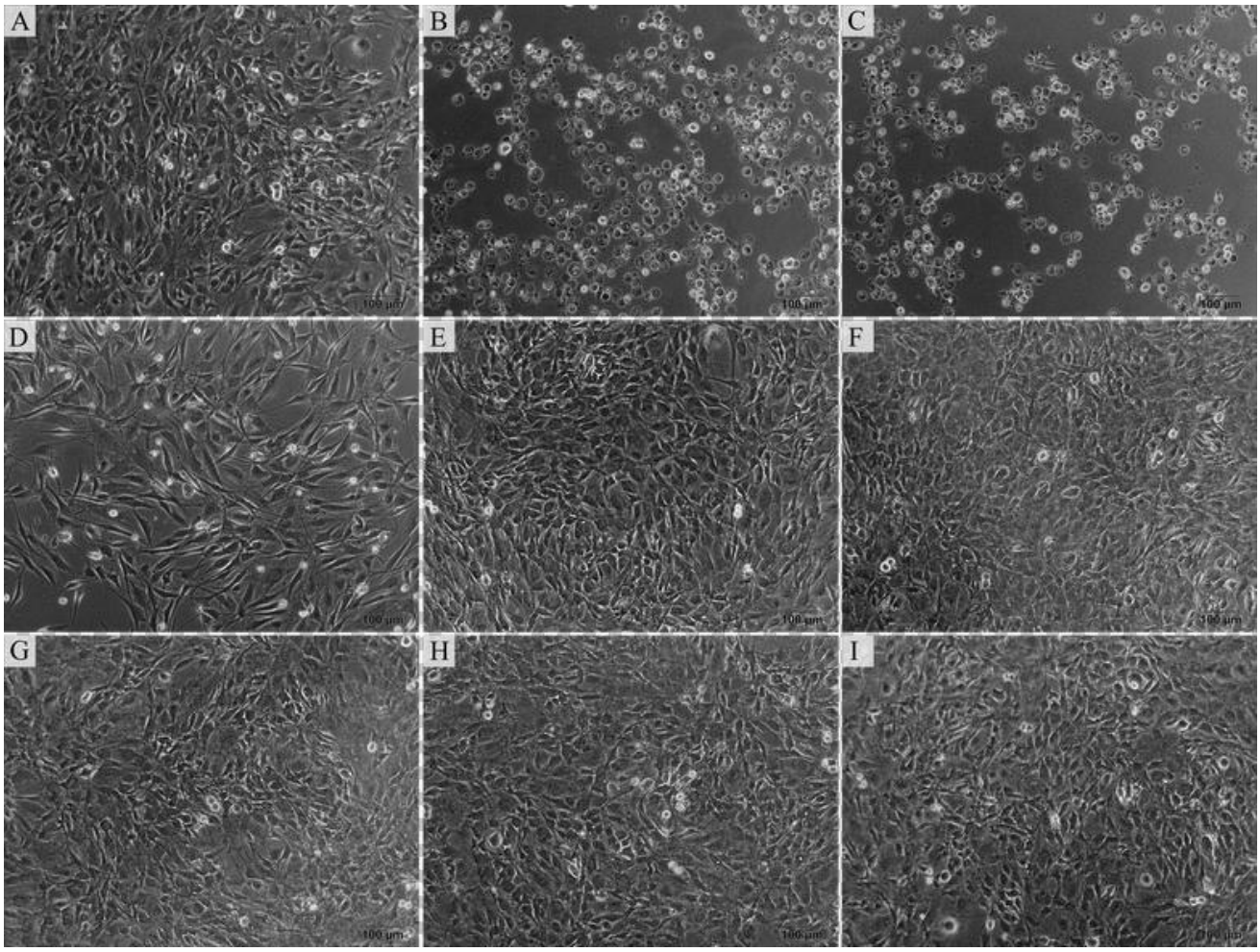

Figure 4 Morphological changes of MC3T3-E1 cells after 24 hours of exposure to the test solutions. (A) Control: polygonal-shaped cells**.** (B-E) Cells treated with 10,000, 7,500, 5,000 or 2,500 µg/ml culture medium of 17% EDTA; respectively. Contracted, spherical morphology and increases in intercellular spaces were observed at the two highest dilutions (B and C) while at 5,000 µg/ml some cells exhibited the normal polygonal morphology; however, decreased cellular density and increased intercellular spaces were also observed (D) meanwhile the normal polygonal morphology of the cells was retained at 2,500 µg/ml (E)**.** (F-I) Cells treated with 10,000, 7,500, 5,000 or 2,500 µg/ml culture medium of 1% IP6; respectively. Cells retained the normal polygonal morphology at the various dilutions of IP6.

Effect on resin adhesion to NaOCl-treated dentin

A significant difference was observed among the groups in the μTBS values ($P < 0.05$). The Tukey test further indicated that the μTBS value obtained after application of 5% NaOCl (51.3 ± 16.4 MPa) for 5 minutes was significantly different from the control (71.6 ± 19.9 MPa) ($P < 0.001$). However, the application of either 17% EDTA for 1 minute (68.5 ± 22.5 MPa) or 1% IP6 for 1 minute (65.5 ± 19.7 MPa) or 30 seconds (64.2 ± 12.5 MPa) resulted in μTBS values that were not statistically significant from the control group ($P= 0.409$) but significantly higher than the NaOCl group ($P \leq 0.005$). An interesting observation under SEM in some debonded specimens is resin-tags formation inside the dentinal tubules in groups treated with 1% IP6 for 1 minute (Figure 5D) and 30 seconds (Data not shown).

Table 1 Mean microtensile bond strength (μTBS) values (MPa ±SD) of experimental groups

Group	Mean (Mpa) ± SD
Control	71.6 ± 19.9[a]
5% NaOCl	51.3 ± 16.4[b]
5% NaOCl /17% EDTA	68.5 ± 22.5[a]
5% NaOCl /1% IP6 for 1 minute	65.5 ± 19.7[a]
5% NaOCl/1% IP6 for 30 seconds	64.2± 12.5[a]

Groups identified by different superscript letters indicate significant statistical difference (one-way ANOVA with Tukey post hoc test, alpha = 0.05). SD, standard deviation.

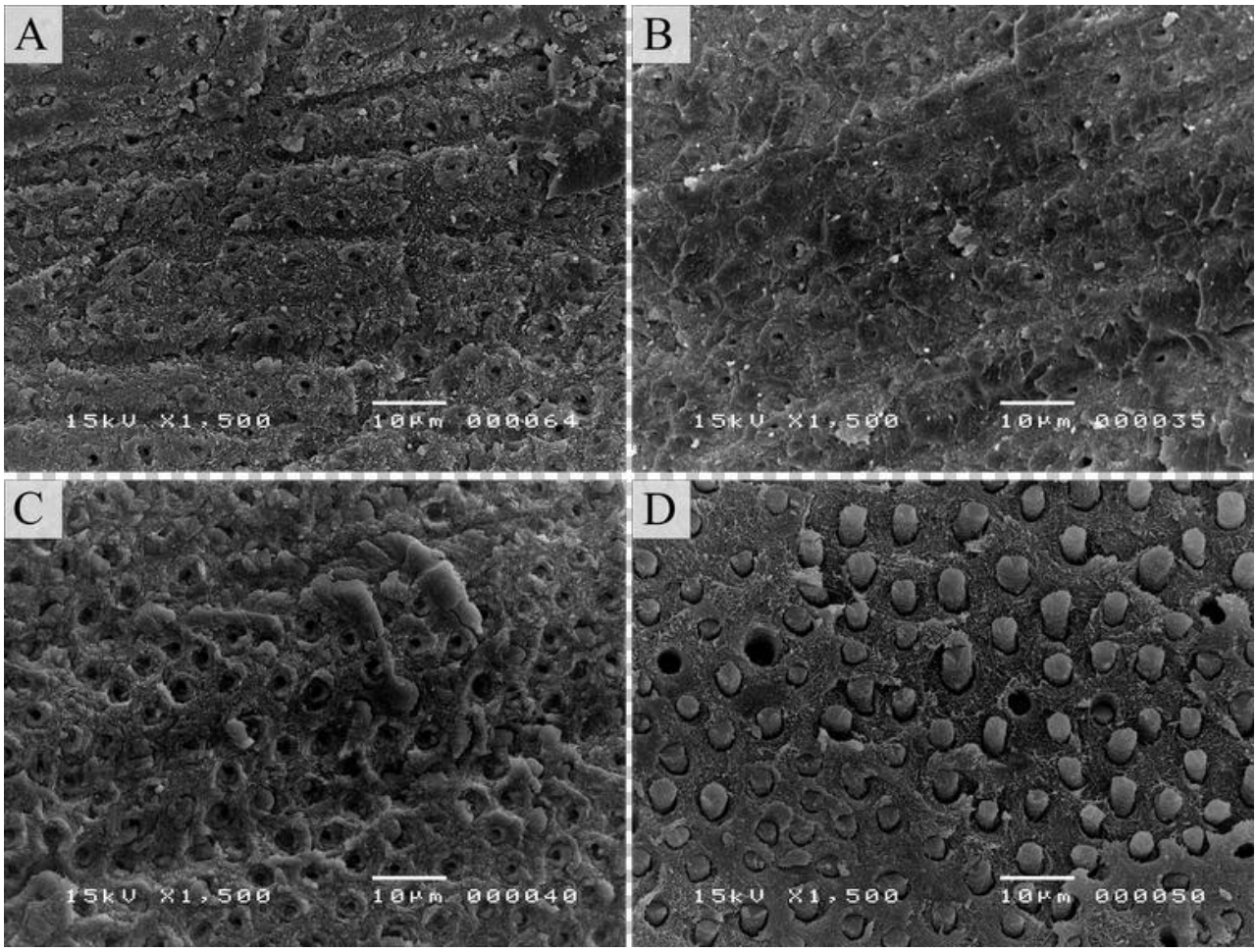

Figure 5 Scanning electron microscopy images of the dentin side of the fractured specimens. (A) Control group, (B) 5% NaOCl treatment for 5 minutes, (C) 1 minute of 17% EDTA treatment of the NaOCl-treated dentin surfaces and (D) 1 minute of 1% IP6 treatment of the NaOCl-treated dentin surfaces. Note the resin tags formation along the surface treated with IP6.

Reaction between the used reagents

The reaction between NaOCl and EDTA was exothermic [43.2 °C] with bubbles formation and a final pH of 7.1 (Figure 6A) while mixing NaOCl with IP6 did not result in either increased temperature or bubbles formation and the final pH was 8.0 (Figure 6B). NaOCl/EDTA mixture was not able to change the color of the paper from brown to white (Figure 7B) while NaOCl/IP6 mixture showed a strong bleaching effect (Figure 7C) that was comparable to pure NaOCl effect on the color of the used paper (Figure 7 A).

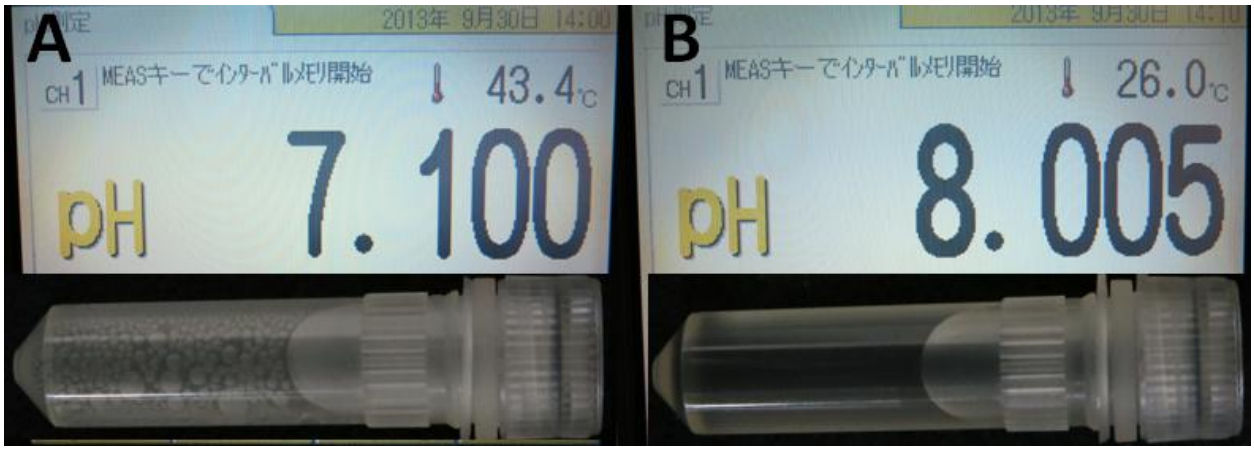

Figure 6 The resultant pH, temperature and bubble formation of the mixture of 5% NaOCl with either 17% EDTA (A) or 1% IP6 (B).

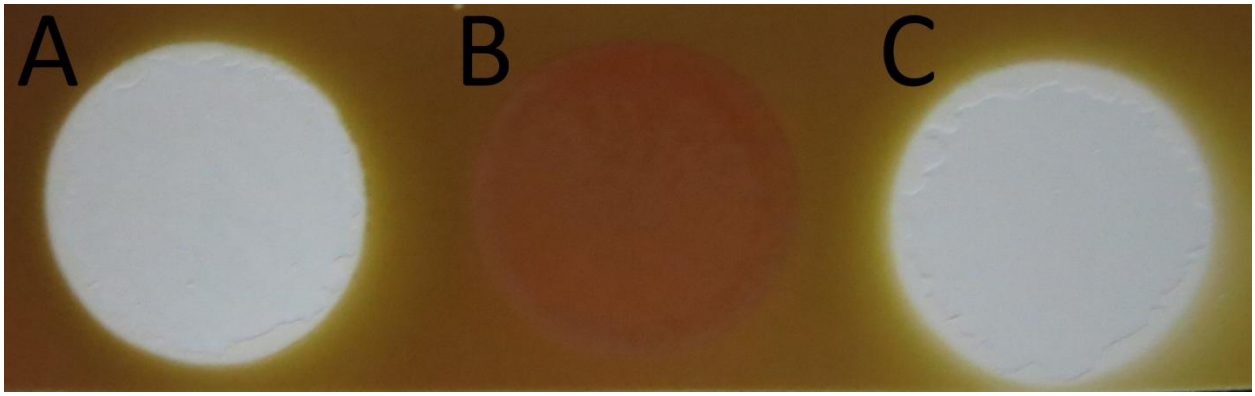

Figure 7 The effect of different mixtures on the color of hematotorpin-stained paper, (A) 5% NaOCl, (B) 5%NaOCl + 17%EDTA and (C) 5% NaOCl + 1% IP6.

CHAPTER FIVE: DISCUSSION

5.1 The Effect of Glutathione on 2-Hydroxyethylmethacrylate Cytotoxicity and on Resin-Dentin Bond Strength

HEMA is a major component of dentin adhesives and the most studied methacrylic dental resin monomer (Nocca *et al*., 2010). Despite the many advantages, it has the risk of cytotoxicity through diffusion into dentin especially when the remaining dentin thickness is minimal (Bouillaguet *et al.*, 1996; Cetinguc *et al.,* 2007). In the present study, cells exposed to HEMA exhibited a significant decrease in cells viability, when compared to the control group. Following exposure to HEMA, the cells lost their polygonal shape, and became smaller, rounded and retracted with increased intercellular spaces. The toxic effect of HEMA to human pulpal cells has been attributed to decreased cellular level of GSH and increased levels of reactive oxygen species (Chang *et al.,* 2005; Huang *et al.,* 2010). There is no consensus regarding this issue; Huang *et al.* stated that reactive oxygen species was produced by HEMA metabolism but not secondary to GSH depletion (Huang *et al.,* 2010). Conversely, Samuelsen *et al.* found that increased levels of reactive oxygen species was the result of reduced cellular level of GSH; they suggested that HEMA binds to intracellular GSH, thus weakening the oxidative defence inside the cells (Samuelsen *et al.,* 2011). In the present study, the cytotoxic effect of HEMA on cells was not shown in the group where 10 mM GSH existed with HEMA. For the 6- and 24-hour exposure times, the cells appeared polygonal in shape with normal size. In the group that contained 5 mM GSH, cells viability was maintained at the 6-hour exposure time while at 24 hours the protective effect of GSH was abolished. 1 mM GSH was ineffective to protect the cells at both exposure times. The mechanism by which GSH inhibits the cytotoxicity of HEMA may be explained in the same way as NAC detoxifies HEMA. The detoxification mechanism of NAC

is based on direct interaction of its cysteine residue with HEMA through an adduct formation, thus hindering HEMA entry into the cells (Nocca *et al.,* 2010). Another suggested mechanism is the reduction of induced reactive oxygen species levels (Spagnuolo *et al.,* 2006). GSH has a significant antioxidant effect and thus can act as an effective free radical scavenger (Samuelsen *et al.,* 2011). However, due to the recent demonstration of NAC-HEMA complex (Nocca *et al.,* 2010) and GSH-HEMA complex (Nocca *et al.,* 2011; Samuelsen *et al.,* 2011), adduct formation is likely to be the principal mechanism for abolishing the cytotoxicity of HEMA. This might explain our finding in which 10 mM GSH was able to protect the cells from 10 mM HEMA-induced up to 24 hours. 1 mM GSH was not able to protect the cells from HEMA-induced damage at both exposure times, while 5 mM GSH showed a protective effect at 6 hours, however, a sharp drop in OD value was observed at the 24-hour exposure time. Different kinetics of HEMA-GSH adduct formation have been observed when the reaction happens in cells or in cell-free system (Nocca *et al.,* 2011), the rate of this adduct formation is higher in presence of cells due to the existence of glutathione S-transferase enzyme that catalyzes the reaction; moreover, HEMA metabolites could increase the rate of the spontaneous formation of complex with GSH. Furthermore, this reaction is both time and concentration dependent; increased intensity of adduct formation was detected with increased reactant concentrations (Samuelsen *et al.,* 2011). We suspect that 5 mM GSH acted as reactive oxygen species scavenger during the 6 hours period thus effectively abolished the cytotoxic effects of HEMA while later HEMA-GSH complex started to form and some HEMA remained free from this interaction due to insufficient molecules of GSH to react with HEMA, and this might explain the low OD value obtained at 24-hour exposure time for the group containing 5 mM GSH. A recent study using mice macrophages reported that the use of glutathione synthase inhibitor (buthionine sulfoximine)

enhanced the toxic effect of HEMA by increasing the percentage of cells in apoptosis and necrosis stages, while glutathione synthase inducer (2-oxothiazolidine-4-carboxylate) opposed HEMA-induced apoptotic cell death (Krifka *et al.,* 2012).

Apart from the need to lower or eliminate the cytotoxicity of dentin adhesives, it is also essential to maintain their desirable bonding properties (Paranjpe *et al.,* 2007b). Tensile strength testing is an important parameter in determining the performance of bonding materials in restorative dentistry (ISO/TS 11405, 2003). In the present study, we tested the μTBS of an etch-and-rinse adhesive system to dentin treated with different concentrations of GSH. Our results demonstrated that treatment of acid-etched dentin with 2%, 5% or 10% GSH did not affect the immediate μTBS of the tested adhesive; however, 5% GSH obtained a bond strength value that was statistically higher than the value obtained by 10% GSH. We proposed the use of GSH with an etch-and-rinse adhesive due to the fact that dentin etching with phosphoric acid increases dentin permeability thus increasing the amount of HEMA reaching the pulp (Rathke *et al*., 2007). The optimal concentration of GSH applied to dentin should be further investigated because too high concentration could jeopardize resin-dentin bonding as the free radical scavenging effect of GSH might be amplified to such an extent that free radical polymerization of the adhesive resin monomers becomes adversely affected, causing decline in the mechanical properties of the polymerized adhesive. A previous study using proanthocyanidin, a flavonoid with free radical scavenging effect, reported similar increases in immediate μTBS when dentin was bonded with 1% or 2% proanthocyanidin incorporated in a dentin adhesive, but with reduction in μTBS when 3% PA was incorporated in the adhesive. This adverse effect has been attributed to the inhibition of resin polymerization by proanthocyanidin (Epasinghe *et al.,* 2012).

In conclusion, within the limitations of this study the results indicated that HEMA was toxic to rat pulpal cells. GSH had a protective effect against HEMA cytotoxicity while having no effect on the immediate bond strength of the used adhesive. The clinical significance of the findings of this study is the possible use of GSH to reduce postoperative hypersensitivity that results from the inflammation and cells death due to leaching of adhesive monomers into the pulp. Further studies should be conducted to evaluate the exact mechanism of GSH detoxification of HEMA and the possible incorporation of GSH in adhesives or resin restorations.

5.2 The Inhibition Effect of Non-Protein Thiols on Dentinal Matrix Metalloproteinase Activity and HEMA Cytotoxicity

This was the first study to evaluate the inhibition of dentinal MMPs by NPSH namely; GSH and NAC, and compare the effectiveness of GSH to NAC regarding HEMA toxicity detoxification. The used NPSH agents were able to decrease the gelatinolytic activity of MMP-2. GSH showed similar effectiveness to NAC in eliminating the cytotoxicity of HEMA on rat pulpal cells.

In this study partial demineralization of dentin by PA resulted in increased gelatinolytic activity of MMP-2. Low-pH environment is known to activate MMPs (Mazzoni *et al.,* 2013); via the cysteine-switch mechanism or by affecting the binding of tissue inhibitors of metalloproteinases to MMPs (Chaussain-Miller *et al.,* 2006). In our study, PA could activate pro- and active forms of MMP-2. Both MMP-2 and MMP-9 are detected in human dentin; however, it is thought that MMP-2 is the major gelatinase (Mazzoni *et al.,* 2007; Niu *et al.,* 2011) and this might explain the strong activity of MMP-2 obtained in this study.

Treatment of demineralized dentin with either GSH or NAC resulted in decreased pro-MMP-2 and active-MMP-2 gelatinolytic activities. Sulfur-containing compounds inhibit MMPs through a variety of mechanisms including i) as component of the general thiol/disulfide redox buffer, ii) as metal chelators and iii) as radical quenchers. However, for thiol antioxidants (GSH, NAC), the in-vitro inhibitory effect is due to the free thiol group of the cysteine moiety (Emara and Cheung, 2006). In the inactive MMP a conserved cysteine residue of the prodomain is coordinated to the catalytic Zn^{+2}, thus forming a cap over the active center blocking the enzyme. For activation, the cysteine residue is displaced; H_2O binds as the fourth ligand to Zn^{+2} and the proteinase becomes active (Koch *et al.,* 2009). It was suggested that GSH interacts with MMP's active-site Zn^{+2}, and this interaction involves a disulfide bond formation. Although GSH has the ability to react with a wide variety of redox-sensitive metal ions, the slow dissociation rate, reflective of the stability of the GSH-Zn^{+2} complex, makes Zn^{+2} interaction unique (Pei *et al.,* 2006). In this study, CHX showed to be effective in decreasing the gelatinolytic activity of both pro-MMP-2 and active-MMP-2. The mechanism of CHX inhibition of MMPs is mainly due to its ability to chelate with Ca^{+2} and Zn^{+2}; however, other mechanisms have been suggested such as interaction with sulfhydryl and cysteine residue present in the active MMPs (Gendron *et al.,* 1999) or even denaturation of proteins at high CHX concentrations (Hjeljord *et al.,* 1973). Osorio *et al*. found that the inhibitory effect of CHX in non-resin infiltrated PA-treated dentin was limited, as CHX binds electrostatically to demineralized dentin collagen thus in presence of other cations it might diffuse out of the collagen matrix; however, its effect might be prolonged in resin-infiltrated collagen where monomers might exert a protective effect on demineralized collagen through immobilization of MMPs (Osorio *et al.,* 2011b).

The need of the use of effective, non-toxic MMPs inhibitors cannot be stressed enough, as the use of PA results not only in MMP activation but also in increased dentin permeability which can lead to the diffusion of toxic inhibitors such as CHX (Lessa *et al.,* 2010) or some adhesive components such as HEMA (Rathke *et al.,* 2007). In this study, HEMA adversely affected cells viability and changed their morphology from being polygonal to become rounded and smaller with the loss of intercellular spaces. Increased reactive oxygen species and/or decreased cellular levels of GSH have been proposed as mechanisms of HEMA toxicity (Chang *et al.,* 2005; Huang *et al.,* 2010; Krifka *et al.,* 2010; Samuelsen *et al.,* 2011) which would inhibit the differentiation of pulpal cells into odontoblasts (About *et al.,* 2002) and induce apoptotic cell death (Chang *et al.,* 2005). In our study the use of either GSH or NAC contributed to effective protection of the cells from HEMA cytotoxicity where the cells retained their normal morphology. There is no agreement on the exact mechanism by which NAC inhibits HEMA toxicity, but several mechanism have been proposed as follows: (i) enhancement of cellular levels of GSH (Yamada *et al.,* 2009); (ii) reduction of reactive oxygen species levels (spagnuolo *et al.,* 2006); (iii) up-regulation of the nuclear transcription factor $_k$B (NK_kB) that is a regulator of anti-apoptotic proteins (Paranjpe *et al.,* 2007a) and (iv) a detoxification mechanism that is based on direct interaction between the cysteine residue of NAC with HEMA thus obstructing HEMA entry into the cells (Nocca *et al.,* 2010). Due to the similarities of chemical structures between NAC and GSH we speculate that GSH provided this survival action for pulpal cells through at least one of the above mentioned mechanisms. Thus, the application of GSH in our study could have directly increased the intracellular levels of GSH, effectively scavenged free radicals through its significant antioxidant property (Fang *et al.,* 2002), modulated the activity of NK_kB (Mihm *et al.,* 1995), or directly interacted with HEMA thus preventing HEMA from

interacting with the intracellular GSH (Samuelsen *et al.,* 2011). In the present study, GSH showed similar effectiveness to NAC in inhibiting the toxic effect of HEMA at both exposure times. GSH+HEMA and NAC+HEMA groups showed better cells viability compared with the control at 24-hour exposure time. In the groups where GSH or NAC existed without HEMA, cells viability was also significantly higher than the control at 24-hour exposure time. It can be concluded that these antioxidants contributed to a more favorable environment for the cells to grow, reflecting that the role of these thiols is not only limited to the interior of the cells but also to the extracellular medium (Schafer and Buettner, 2001). In the present study, CHX was shown to be toxic to rat pulpal cells, and not of a surprise its use with HEMA did not reverse the latter cytotoxicity. Recently, CHX was reported to inhibit growth and collagen synthesis of osteoblastic cells, these effects were considered not cell type specific, intriguingly CHX cytotoxicity on these cells was related to depletion of intracellular GSH (lee *et al.,* 2010), this would raise the need for future studies exploring the possible role of GSH and NAC in protecting the pulpal cells from CHX-mediated cell death. It is noteworthy to mention that despite the worries about CHX toxicity, this agent has being successfully used under restorations for many years. The ability of CHX to bind to demineralized dentin (Kim *et al.*, 2010b) and to promote dentin remineralization (Kim *et al.,* 2011) can limit its diffusion thus lowering its toxic effect.

The limitations of this in vitro study include the use of powdered dentin where mechanical stress during preparation can result in the autolytic activation of the bound latent enzymes thus the proteolytic activities observed may simply reflect artefacts during specimen preparation (Mazzoni *et al*., 2006) and the use of zymography which has limited profiling with no possibility of proteinases activity quantification (Klein *et al*., 2009). Within the aforementioned limitations, the results of this study support the use of NPSH on PA-treated

dentin to inhibit MMPs that are activated after dentin treatment with PA, a step that would probably increase the durability of resin-dentin bonds; moreover, the use of such highly non-toxic compounds not only can enhance the viability of pulpal cells but also protect them from the induced damage caused by HEMA thereby reducing possible postoperative hypersensitivity that might be caused by this monomer leaching into the pulp. One might argue that CHX has the antimicrobial property in its favor over these thiol compounds, but it is not surprisingly to know that both GSH and NAC have some antimicrobial effectiveness against gram-positive and gram-negative bacteria (Perez-Giraldo *et al.,* 1997; Zhang and Duan, 2009; Quah *et al.,* 2012). However, further in vitro and in vivo studies have to be performed to examine the effect of NPSH on other properties of adhesion such as bond strength and mechanical properties with the possible incorporation of these compounds in restorative materials.

5.3 The Effect of Phytic Acid Used as Etchant on the Bond Strength, Smear Layer and Pulpal Cells

This was the first study to evaluate the effect of IP6, used as etchant, on dentin bond strength, smear layer removal and viability of pulpal cells. The findings of this study demonstrated that 1% IP6 applied for 1 minute, 30 seconds or 15 seconds significantly enhanced dentin bond strength, effectively removed the smear layer and had minimal effect on the viability of pulpal cells.

Dentin etching with PA is performed to remove the mineral phase of the smear layer and expose a collagen network that will be later infiltrated by the adhesive that fills the gaps between the collagen fibrils forming the hybrid layer (Vaidyanathan and Vaidyanathan, 2009). PA creates

a delicate collagen network that is depleted of hydroxyapatite and that would collapse upon drying, thus hindering effective infiltration of the adhesive (Prati *et al.,* 1999; El Feninat *et al.,* 2001). Several ways have been proposed to overcome this problem in the two-step etch-and-rinse adhesives; among the earliest was the introduction of "wet-bonding technique" with either water (Kanca, 1992) or ethanol (Pashley *et al.*, 2007). These two approaches have some drawbacks. For water wet-bonding, it is difficult to determine the degree of moisture to be left on dentinal surface (Hitmi *et al.,* 2002); moreover, shrinkage of collagen has been shown to occur, even under moist conditions (Pashley *et al.,* 2000; Nakajima *et al.*, 2002), because the wet collagen is very complaint and soft, and thus can easily shrink when the solvent is evaporated (Pashley *et al.,* 2007). Ethanol wet-bonding is considered as a concept rather than a technique, which is difficult to be applied clinically (Osorio *et al.,* 2010). The use of glutaraldehyde has been proposed as a way to increase the bond strength and improve collagen stability in etched-dentin; however, there is always concern about the toxicity of this chemical cross-linker (Macedo *et al.,* 2009).

In this study, IP6 proved to be an effective agent for removing the smear layer; this effect is caused by its ability to chelate with Ca^{+2} (Cowieson *et al.,* 2006). Dentin surfaces treated with IP6 were clean of smear layer and smear plugs, dentinal tubules were open, and intertubular collagen was exposed. IP6-etched dentin showed a resin-dentin bond strength that is higher than the resin-dentin bond strength of PA-etched dentin. The increase in bond strength might be attributed to two mechanisms: [a] the ability of IP6 to from complexes with Ca^{+2}, these complexes are insoluble in a pH above 4 (Grynspan and Cheryan, 1983). As a result of the high buffering capacity of dentin (Camps and Pashley, 2000), we expect the pH of IP6 to increase upon neutralization with dentin, and thus a type of insoluble complexes is created with hydroxyapatite that might provide some stability for the exposed collagen, [b] IP6 acts as a

cross-linker (Lee *et al*., 2011; Ravichandran *et al.,* 2013) that could mechanically strengthen the collagen and prevent their collapse. The interaction between IP6 and protein is reported to be direct electrostatic interaction (Cheryan, 1980); the cross-linking effect of IP6 results from the bonding of anions of IP6 with the cations of proteins (Ravichandran *et al.,* 2013). It is known that the dentinal collagen would have a positive net charge upon exposure to acidic solutions (Nezu and Winnik, 2000; Zhang *et al.,* 2005) and thus we speculate the aforementioned interaction to occur between IP6 and dentinal collagen. The increase in mixed mode of failures at the adhesive-resin interface for the IP6-etched group reflects the higher bond strength values and may indicate that the hybrid layer has been strengthened by the use of IP6.

Little is known about the effect etching agents on pulpal cells. Clinically, these agents are applied on dentin which has the ability to dilute them. It has previously been shown that 10% of the amount of acids applied on a 0.6 mm thick dentin is taken up by dentin and only 1% can diffuse through the complete thickness of dentin (Camps and Pashely, 2000); thus, in the present study, 1/10 and 1/100 of the concentrations applied in the bond strength test were used to test the effect on pulpal cells. The findings of this study demonstrated that 3.7% and 0.37% PA were toxic to pulpal cells as demonstrated by the low OD values obtained for the groups treated by these two concentrations of PA. PA is known to induce DNA damage in human lymphocyte (Yilmaz *et al.,* 2012). It has been reported that prolonged etching time resulted in immediate failure of the microcirculation in rat dental pulp (Ivanyi *et al.,* 2001). The toxic effect of PA on fibroblasts was attributed to extracellular acidosis created by the low pH levels of this agent (Navarro-escobar *et al.,* 2010).

After 6 hours of exposure, IP6 (0.1% and 0.01%) did not show a negative effect on pulpal cells; in contrast, enhanced viability of the cells was observed. At the 24-hour exposure time

0.01% did not show any significant difference from the control while 0.1% caused a minimal decrease in the OD value; however, it was statistically significant from the control. These results can be interpreted by the ability of IP6 to interact with iron. IP6 can reduce the level of oxidative stress in cultured cells through chelation with iron, a metal that is known to catalyze the formation of hydroxyl-radicals (Xu *et al*., 2008). In this study, fetal bovine serum (which contains elevated levels of iron stores) was used for cell culture (Kakuta et al., 1997). It was reported that IP6 has a dual role in cell culture; besides its action as iron chelator, it is also considered as good source of phosphate for cells (Rasmussen and Toftulund, 1986). The decrease in OD value at 24-hour exposure time to IP6 could be linked to the depletion of iron inside the cells as a result of the lack of iron available in the culture medium. Despite its toxic effect, iron is an essential component in culture medium as a promoter of protein production (Bai *et al.,* 2011).

Within the limitations of this study, it was concluded that etching of dentin with IP6 enhanced the bond strength of etch-and-rinse adhesive to dentin, efficiently removed the smear layer, and had minimal effects of pulpal cells. Further experiments are warranted to evaluate the effect of IP6 on the long-term bond strength and mechanical properties of dentin. The results of this study may provide insights into the development of a new natural compound that has the potential to replace PA for dentin etching, a step that would simplify the clinical protocol used for etch-and-rinse adhesive where an additional collagen cross-linker or ethanol is necessary after PA etching to ensure effective monomer infiltration in the exposed collagen. Moreover, postoperative sensitivity encountered with etch-and-rinse adhesives could be brought to a lower level with the use of IP6.

5.4 Phytic Acid: An Alternative Root Canal Chelating Agent

Chelating agents play an integral part of root canal therapy. The choice of an irrigation solution should take in consideration its smear layer removal ability, biocompatibility and the effect on adhesion of resin-based materials to dentin. In this study, IP6 was able to remove the smear layer from flat dentin surfaces and instrumented root canals, did not show negative effect on the viability and ALP activity of osteoblast-like cells and enhanced resin adhesion to NaOCl-treated dentin surfaces.

Removal of the smear layer from instrumented root canal surfaces before obturation is a recommended step that is usually done by the use of 17% EDTA for 1 minute. The results of this study is in accordance with the literature, in which EDTA was effective to remove the smear layer from the surface of instrumented root canals, this ability resides in the property of ionized EDTA to chelate Ca^{+2}. The optimum pH for EDTA effectiveness is in the range of 6-10 because higher ratio of ionized to non-ionized molecules can be achieved at higher pH; however, the availability of Ca^{+2} for chelation decreases at higher pH. When EDTA forms a complex with Ca^{+2}, a proton is released resulting in decreased pH of the environment in which EDTA loses its efficiency (O'Connell *et al.,* 2000). In this study, IP6 proved to be effective in removing the smear layer from NaOCl-treated flat dentin surface and instrumented root canals. IP6 is highly negatively charged molecule that has affinity to Ca^{+2} (Luttrell, 1993; Torres *et al.,* 2005). Flat dentin surfaces treated with 1%IP6 were cleaner with wider openings of dentinal tubules when compared to dentin surfaces treated with EDTA. On root canal surfaces, the effect of both IP6 and EDTA in cleaning the apical third was less than that in the middle third, and this is attributed to the anatomy of the former region. The pH of 1% IP6 solution is around 1.2 and this acidity

contributes to better Ca^{+2} extraction. Thus, the acidity and chelate function of IP6 make it an effective smear layer removal agent.

Taking into account that osteoblast cells are necessary element for periapical healing, it is important to assess the effect of agents used inside the root canal on the viability and ALP activity of these cells as the use of these agents holds the risk of their extrusion beyond the apical foramen. To mimic the clinical situation in which the extruded agent gets diluted once in contact with the periapical tissue, concentrations lower than the recommended one for clinical use were used in this study. In the present study, the three highest concentrations of EDTA caused a dramatic decrease in cells viability affecting their morphology while lower concentrations were non-toxic. ALP activity is correlated with the mineralization ability of the osteoblast cells (Beck *et al.*, 1998); 500 µg/ml of 17% EDTA caused a significant suppression of ALP activity. The negative effect of EDTA on periapical cells and on the immune reaction has been previously studied. This agent was reported to disrupt the cell membrane structure and the function of macrophages thus interfering with the healing process (Amaral *et al.*, 2007).

The presence of IP6 in the culture medium did not affect the viability, morphology or the ALP activity of the cells in all tested concentrations. IP6 was reported to have a double role in cell culture; as an iron chelator and source of phosphate for cells (Rasmussen and Toftlund, 1986). IP6 protects the cells from oxidative injury through binding to iron, a metal that catalyzes the formation of hydroxyl radicals (Xu *et al.*, 2008). Thus the results obtained in the present study might be partially attributed to this property.

The effect of irrigation solutions used in endodontic treatment on resin adhesion to dentin has become an issue of interest not only because of the development in dentin bonding systems

that led to increased restoration of endodontically-treated teeth with resin composite (Ozturk and Ozer, 2004) but also due to the need to accomplish the definitive restoration of these teeth as soon as possible after obturation (Saunders and Saunders, 1994); this implies that dentin which will receive the bonding is exposed to these irrigants (Ozturk and Ozer, 2004). In this study, the use of 5% NaOCl resulted in decreased tensile bond strength of the one-step self-etch adhesive to dentin. Several previous studies have reported the negative effect of NaOCl on resin adhesion to dentin and that was mainly attributed to the inhibition of resin polymerization caused by NaOCl (Nikaido *et al.*, 1999; Weston *et al.*, 2007). The use of either EDTA or IP6 proved to abolish this deleterious effect, this action could be related to the demineralising effect of these agents, thus exposing the underlying dentin in which the effect of NaOCl is not of significance. The mode of failure of the fractured specimens revealed the presence of resin-tags that formed in most of the dentinal tubules on specimens treated with IP6, a pattern that was not observed in the other groups, this reflects the better ability of IP6 in opening dentinal tubules, a property that could be of importance in root canal treatment, allowing superior penetration of inter-appointment medicament or sealer into root canal dentinal tubules which results; respectively, in better disinfection and sealability.

The reactions between different irrigants used in endodontic gained special interest (Santos Junior *et al.*, 2013). These reactions can happen even after flushing the irrigant out of the canal because dentinal tubules would still hide enough amount of it to react with the next irrigant (Bui *et al.*, 2008). 1% IP6 showed less reactivity with NaOCl when compared to 17% EDTA as the reaction was non-exothermic, with no bubble formation and the mixture was able to change the color of the stained paper into white while the mixture of NaOCl and EDTA did not have this

effect and this can be explained by the depletion of the active chlorine after mixing NaOCl with EDTA.

The findings of this study showed the potential of IP6 to be used as a chelating agent in root canal treatment. IP6 proved to be an effective agent in removing the smear layer and restoring the bond-strength to NaOCl-treated dentin while being biocompatible to osteoblast-like cells and having less reactivity with NaOCl. Further studies should be conducted to evaluate its effect in vivo experiments before recommending it for the clinical use in endodontic practice.

CONCLUSIONS

- The use of GSH in controlled concentrations had no effect on the immediate bond strength of the used etch-and-rinse adhesive to dentin.
- GSH was effective to abolish the toxicity of HEMA on pulpal-like cells in a dose-dependent manner and its effectiveness was comparable to NAC.
- Both GSH and NAC decreased the gelatinolytic activities of dentinal MMPs that were activated by PA.
- IP6 was effective in etching smear-layered dentinal surfaces and exposing the collagen network. It enhanced the bond strength of the used etch-and-rinse adhesive to dentin and showed minimal effect on pulpal-like cells thus it has the potential to replace PA in etch-and-rinse adhesives.
- IP6 was effective in removing the smear layer from NaOCl-treated dentinal surfaces and instrumented root canals. It also restored the bond strength of resin adhesive to NaOCl-treated dentinal surfaces. It showed biocompatibility to osteoblast-like cells and was less reactive to NaOCl when compared to EDTA. Taken these findings together; IP6 can possibly replace EDTA as a chelating agent in root canal treatment.

RECOMMENDATIONS

- Further experiments should be conducted to evaluate the effect of NPSH incorporation into resin-adhesives on the polymerization behavior of resin adhesives.
- In vivo studies are warranted to explore the clinical effectiveness of NPSH added to resin adhesives in preventing resin toxicity on pulpal cells, inhibition of dentinal MMPs and enhancing resin-dentin bond durability.
- In vivo studies are warranted before any firm conclusions can be drawn about the use of IP6 as an etching and/or chelating agent.

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