EFFECT OF AN ADHESIVE RESIN ON DENTAL PULP CELL APOPTOSIS AND VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION

by

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This thesis is dedicated to my dearly beloved husband, Luis Enrique, from whom I receive every day the support, friendship and love that were essential for me through these years. Thank you for always being by my side.

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INTRODUCTION

Angiogenesis is a fundamental biological process by which new blood vessels are formed from pre-existing microvasculature. It occurs normally during physiologic and reparative processes such as ovulation and wound healing (Folkman, 1992). Angiogenesis is a highly regulated process that is turned on for brief periods of time by the presence of angiogenic factors and then may be completely blocked by specific inhibitors. Defining the mechanisms whereby angiogenesis is triggered might ultimately lead to better ways to regulate the process therapeutically.

Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis in a variety of physiological and pathological biological processes. VEGF was initially termed vascular permeability factor (VPF). Due to its ability to enhance vascular permeability, Dvorak and colleagues, 1985) discovered its function, as an inducer of vascular permeability. Furthermore, VEGF induces endothelial cell proliferation, promotes cell migration, mitogenesis, and edema, (Ferrara, 1996). The synthesis of VEGF is regulated by hypoxia-mediated control of gene transcription, alternative mRNA splicing, and post-translationally (at the protein level).

Basic fibroblast growth factor (bFGF) is one of nine structurally-related polypeptides. It was the first angiogenic cytokine originally purified from bovine pituitary gland, based on heparin affinity chromatography (Esch *et al.*, 1985), and sequenced and characterized as an angiogenic factor (Abraham *et al.,* 1986). Basic FGF stimulates extracellular matrix (EC) proliferation *in vitro*, induces angiogenesis *in vivo*, and is frequently present at sites of capillary growth (Schweigerer *et al.*, 1987, Folkman *et al.*, 1987). Araki and colleagues, 1990, were the first group to implicate FGF as a

survival factor for endothelial cells. Enhanced activity of protein kinase C was associated with the ability of bFGF to protect endothelial cells against apoptosis induced by growth factor deprivation or ionizing radiation *in vivo* and *in vitro* (Araki *et al.*, 1990).

Another important pro-angiogenic factor is interleukin 8 (Il-8). It is secreted by many specific cell types (macrophages and others) and induces proliferation, cell migration and organization into sprout-like structures that mimic stages in the development of microvessels *in vivo* (Streieter & Polverini 1992) In previous studies it has been demonstrated that Il-8 induces a rapid increase in endothelial cell number that had gradually declined to levels that were at or below levels encountered in control groups (Folkman and Shing, 1992; Risau, 1997). This finding is presumably due to Il-8's lack of ability to upregulate and sustain endothelial cell survival (Nör *et al.,* 1999).

The role of growth factors in mediating cellular responses to injury in dental pulp still remains unclear. Matsushita *et al.,* (1999) investigated whether VEGF production by pulp cells was regulated by lipopolysaccharides during the pathogenesis of pulpitis. This study suggested that enhanced production of VEGF in human pulp cells induced by lipopolysaccharides is through a CD14-dependent pathway, which requires new protein synthesis and is mediated in part during AP-1 activation. The authors hypothesized that VEGF induces vascular permeability, proliferation, differentiation of pulp cells and also chemotaxis of monocytes and macrophages. Matsushita *et al.,* (2000) attempted to further clarify the role of VEGF in human dental pulp cells and pulp tissue. They investigated the effects of VEGF on the chemotaxis, proliferation, and differentiation of human dental pulp cells. The authors found that VEGF induced a strong chemotactic response in human dental pulps cells in a dose-dependent manner. The results suggested that VEGF was

produced by human pulp cells and acted directly upon human dental pulp cells in an autocrine manner. Furthermore, VEGF may promote chemotaxis, proliferation and differentiation via the utilization of kinase insert domain-containing receptor and in part through AP-1 by increasing expression of c-fos. Local angiogenesis at the injury site in the dentin-pulp complex appears to be critical for successful pulpal repair. Roberts-Clark and Smith (2000) have shown that the dentin matrix contains angiogenic factors, and that their release from the matrix after injury could make an important contribution to the overall reparative response of the dentin-pulp complex.

Inhibitors of VEGF have been used also to downregulate vessel growth when unwanted or over-exuberant angiogenesis and/or edema occurs. VEGF-induced effects are mediated through receptor tyrosine kinases, specifically the Flt-1 and Flk-1/KDR receptors (Neufeld *et al.,* 1999). Activation of the Flk-1/ KDR receptor is associated with endothelial cell proliferation, whereas the Flt-1 receptor appears to be more involved in the regulation of cell-cell or cell-matrix interactions. Anti-VEGF antibody or tyrosine kinase inhibitors such as PTK787 inhibitor (Novartis, Basel, Swiss) have been successfully used in phase II and III clinical trials to downregulate pathological angiogenesis.

Previous studies have demonstrated that the application of an adhesive resin directly over the pulp tissue results in enhanced local vascularization and lack of dentin bridge development. The increased vascularization and edema observed after direct pulp capping with adhesive resin might result in irreversible tissue damage. A unique feature of the pulp is that it is confined within rigid, non-expandable dentin walls. Therefore, increases in microvessel density and permeability, which have been associated with

increased intra-pulpal pressure (Van Hassel, 1971), might result in pulp necrosis. However we still do not know which angiogenic factor mediates adhesive resin-induced pulp vascularization. In addition to the effect of adhesive resins on pulp vascularity, it has been also reported that they can induce death of pulp cells *in vitro* (Costa *et al.* 1999) and lack of dentin bridging *in vivo* (Hebling *et al.*, 1999). However, the mechanism of adhesive resin-induced death of pulp cells and its effect on pulp cell cycles are still not completely understood.

Statement of Purpose

The specific aims of this study are:

1) To evaluate the effect of an adhesive resin (Single Bond), or one its hydrophilic monomers (HEMA), on the viability and cell cycle of odontoblasts-like cells, undifferentiated pulp cells, and macrophages.

2) To examine if vascular endothelial growth factor (VEGF) is upregulated in pulp cells exposed to an adhesive resin or HEMA *in vitro*, and to determine which cells synthesize this factor.

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Chapter II

EFFECTS OF AN ADHESIVE RESIN ON APOPTOSIS AND CELL CYCLE OF DENTAL PULP CELLS AND MACROPHAGES

Abstract

In vivo studies have demonstrated that direct pulp capping with adhesive resin systems results in pulp inflammatory responses and absence of dentin development of a dentin bridge. The high oxygen tension and humidity observed at the site of pulp exposure might hinder the polymerization of an adhesive resin applied directly to the pulp and enhance its cytotoxicity. Here, we hypothesized that the lack of dentin bridging might be due to adhesive resin-induced apoptosis of cells responsible for dentin regeneration and dental pulp healing. To evaluate the effect of an adhesive resin on viability and cell cycle of these cells, odontoblast-like cells (MDPC-23), undifferentiated pulp cells (OD-21), or macrophages (Raw) were exposed to Single Bond or 2-hydroxyethyl methacrylate (HEMA) and evaluated by Trypan Blue and flow cytometry. Single Bond specimens were light-cured for 0, 10 or 40 s and placed over a 0.4-μm-pore size polycarbonate insert for cell cultures. Alternatively, cells were exposed to 0-1000 nM HEMA solubilized in culture medium. We found that unpolymerized (0 s) or partially polymerized (10 s) Single Bond induced apoptosis of 90-100% MDPC-23, OD-21, and macrophages within 24 h. In contrast, Single Bond polymerized for 40 s induced

apoptosis of 10%, 10.4%, and 22.6% cells respectively. MDPC-23, OD-21 and macrophages exposed to Single Bond polymerized for 0 s or 10 s showed a significant shift in cell cycle compared to untreated cells, since most cells entered sub-G_0/G_1 after 6 or 9 h. In contrast, cells exposed to Single Bond polymerized for 40 s did not show significant cell cycle changes. HEMA did not affect viability of the cells evaluated here. We concluded that unpolymerized or partially polymerized adhesive resin induces apoptosis or cell cycle arrest of cells involved in the process of dentin regeneration. This might explain, in part, the lack of dentin bridge observed in teeth treated with direct pulp capping procedure with adhesive resins.

Introduction

Adhesive resin systems are used to reduce microleakage, to enhance retention, and to decrease post-operative sensitivity of composite resin restorations. *In vivo* studies have demonstrated that the direct application of adhesive resins to the site of pulp exposure, or to a thin layer of dentin (≤0.5 mm) induces dilatation and congestion of blood vessels, inflammation, and pulp abscesses accompanied or not with tooth sensitivity (Hebling *et al.,* 1999). Importantly, no dentin bridge can be seen in the majority of teeth treated with direct pulp capping with adhesive resin (Pereira *et al.,* 2000). The observed lack of dentin bridge might render the pulp more susceptible to inflammation and/or necrosis mediated by bacterial contamination if microleakage is observed at the resin-tooth interface (Costa *et al.,* 2000).

Complete polymerization of adhesive resins is largely unachievable during direct pulp capping procedures due to the high humidity and oxygen tension of the pulp tissue (Paul *et al.,* 1999). Studies have demonstrated that oxygen inhibits or prevents complete polymerization of adhesive resin monomers (Rueggeberg *et al.*, 1990). In carious teeth, in which the remaining dentin thickness is ≤0.5 mm, it has been demonstrated that humidity may prevent complete polymerization of adhesive resin (Hamid *et al.,* 1990; Gerzina *et al., 1995, 1996*). Once dentin has been cut for a cavity preparation in a vital tooth, the water content within the intratubular compartment is free to move outward. Even in the absence of fluid shifts, the water-filled tubules provide diffusion channels for noxious substances, such as unpolymerized dentin bonding agent, which might diffuse inward toward the pulp (Hanks *et al.*, 1992, 1994; Pashley, 2000). The diffusion of unpolymerized dentin bonding agents becomes significant clinically when these materials

permeate across dentin of a fresh cavity preparation and the concentrations are high enough to produce a biological effect in dental pulps (Pashley *et al.*, 1988, 1996).

All cells have the ability to die through activation of an internally encoded suicide program, which initiates a characteristic form of cell death called "apoptosis", (Thompson *et al., 1995*). On the contrary necrosis is a pathological form of cell death that results in disruption of cell membrane, release of cell components to the extracellular matrix, and inflammation. The rate of cell division is a tightly regulated process that is intimately associated with tissue turnover, differentiation, and wound healing. Cell proliferation involves two easily recognized and coordinated events: DNA duplication, and physical cell division into two daughter cells. Cytotoxicity is frequently translated in changes in cell cycle patterns (Darnell *et al.,* 1990). When cytotoxic stimuli are intense, cells may escape from the cell cycle and undergo programmed cell death (apoptosis). Apoptotic cells are identifiable by flow cytometry as a sub G_0-G1 population after staining with propidium iodide (Nör *et al.*, 2002).

Dentin regeneration is a process characterized by the mineralization of a dentin bridge at the site of pulp exposure (Pashley *et al.*, 1988). This process is mediated by odontoblasts, which are terminally differentiated cells that do not proliferate *in vivo* (Schroder, 1985). Undifferentiated stromal pulp cells are believed to be responsible for the replacement of dead odontoblasts (Fitzgerald 1990; Smith 1995; Tziafas 1995). Macrophages are considered important orchestrators of wound healing responses (Polverini, 1995). It has been previously reported that direct pulp capping with adhesive resin does not result in dentin bridge formation even in absence of bacterial contamination (Hebling *et al.,* 1999) Here, we hypothesized that the lack of dentin bridge

might be explained in part by adhesive resin-induced apoptosis of cells that are involved in dentin bridge organization (*i.e.* odontoblasts or undifferentiated pulp cells) and wound healing (*i.e.* macrophages). The purpose of this study was to evaluate the effect of an adhesive resin (Single Bond), or one of its hydrophilic monomers (HEMA), on the viability and cell cycle of odontoblast-like cells, undifferentiated pulp cells, and macrophages.

Materials and Methods

Cell Culture

An *in vitro* system was designed to study if dental pulp cell viability is affected by exposure to 2-hydroxyethyl-methacrylate (HEMA) or to an adhesive resin. Mouse odontoblast-like cells (MDPC-23, gift from C.T. Hanks), undifferentiated mouse pulp cells (OD-21, gift from C.T. Hanks), or mouse macrophages (Raw, ATCC, Manassas, VA) were cultured at 37°C in a humidified CO_2 incubator. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% Fetal Bovine Serum FBS, (Gibco), 250 μg/ml L-glutamine (Gibco), 125 units/ml Penicillin (Gibco), and 125 μg/ml Streptomycin (Gibco). Cells were plated in 12-well-plates, and 0, 10, 100, or 1000 nM of HEMA (batch # 868-77-9, Aldrich Chemical Company, St. Louis, MO) and were added to the culture medium in triplicate wells per condition. Experiments using the adhesive resin Single Bond (3M, Minneapolis, MN) were performed by culturing the cells in 12-well plates and placing the specimens over permeable membrane inserts (0.4 μm pore size, Corning, New York, NY) to prevent direct physical interaction between adhesive resin and cells.

Specimen Preparation

Single Bond discs measuring 5 mm x 2 mm were prepared under sterile conditions with a prefabricated mold. The specimens were polymerized with an Optilux 401 light-curing unit (Demetron, Kerr, Danbury, CT) calibrated at 800 mW/cm^2. The light-curing times were as follows: 10 s, partially polymerized adhesive resin; and 40 s, polymerized adhesive resin. All specimens were weighed in a calibrated electric balance

and only those weighing 11 ± 1 mg were utilized. Unpolymerized (0 s light-curing time) specimens (11 ± 1 mg) were used as positive control, and untreated cells were used as negative controls.

Trypan Blue Exclusion Assays

Cell viability was assessed using trypan blue exclusion assay technique, which measures integrity of the cell membrane (Freshney). After a 0-24 h exposure either to HEMA or Single Bond, MDPC-23, OD-21, or Raw macrophages were retrieved with a solution of 0.25% trypsin supplemented with 1 mM EDTA (Gibco). Aliquots of harvested cells from each well were resuspended in 0.4 % trypan blue dye. The number of necrotic cells and the total number of cells was determined from triplicate wells for each condition by means of a hemacytometer with an optical microscope at 100x.

Flow Cytometry Assays

Flow cytometry was used to evaluate the effects of Single Bond or HEMA on MDPC23, OD21, or macrophage apoptosis and cell cycle. The same experimental conditions described above were used here, except that cells analyzed by flow cytometry were cultured in 6-well plates and treated with twice the volume of HEMA or by using 2 Single Bond discs in each well. After a 0-24 h exposure to Single Bond or HEMA, both attached and floating cells were collected for flow cytometry. Harvested cells were centrifuged at 800-rpm, at 4°C for 5 minutes in 12 x 75 mm tubes. The culture medium was aspirated; and cell pellets were resuspended with 2 ml of ice-cold phosphate-buffered saline (PBS). Samples were centrifuged again, PBS was aspirated, and cell pellets were

resuspended in 50 μl/ml propidium iodide (Sigma, St Louis, MO), O.1% sodium citrate (Sigma), 0.1% Triton X (Sigma), 100μg/ml Rnase A (Sigma), in Milli-Q-water. Immediately after the propidium iodide solution was added, the samples were incubated for 30 minutes in the dark at 4^0C, before flow cytometric analysis.

Statistical Analyses

The statistical analyses of the data were performed by Student's t-test and one-way ANOVA with Sigmastat 2.0 software (SPSS, Chicago, IL); and to isolate the groups that differ from others we used Tukey's tests multiple comparisons. Statistical significance was determined at the $p \leq 0.05$ level.

Results

Degree of Adhesive Resin Polymerization Affects Cell Viability

To examine the effect of the degree of polymerization of an adhesive resin on pulp cells and macrophages viability, we exposed these cells to discs of Single Bond resin that were light-cured for 10 s (partially polymerized), or 40 s (polymerized), or a drop of 0 s (unpolymerized). We observed that unpolymerized and partially polymerized Single Bond rapidly induced necrosis of MDPC-23, OD-21, and Raw Macrophages (Figure 2.1). Nearly 100% of all cell types evaluated were considered dead (positive for trypan blue) 3 hours after exposure to unpolymerized Single Bond, while around 60% of MDPC-23 and OD21, and 80% of macrophages were viable after a 3-hour exposure to partially polymerized Single Bond. After 9 hours, only 20% of MDPC-23 and OD-21, and essentially no macrophages were found viable upon exposure to partially polymerized Single Bond (Figure 2.1) In contrast, when these cells were exposed to polymerized Single Bond their viability was undistinguishable from untreated controls ($p>0.05$) at all time periods evaluated. These data demonstrate a direct correlation between degree of polymerization of the bonding agent and the viability of dental pulp cells and macrophages.

Adhesive Resin Induces Apoptosis of Pulp Cells and Macrophages

To understand the mechanism of cell death induced by an adhesive resin, we exposed MDPC-23, OD-21, and macrophages to Single Bond as above and performed flow cytometry after propidium iodide staining. Unpolymerized Single Bond is a strong inducer of apoptosis since nearly 100% of MDPC-23, OD-21 or Macrophages were

apoptotic after a 12 or 24-hour exposure ($p<0.001$) (Figure 2.2). Exposure to partially polymerized Single Bond induced apoptosis of approximately 50% MDPC-23 ($p<0.05$) and 50% OD-21 ($p<0.05$) after 12 hours (Figure 2.2). This effect was intensified at 24 hours, when about 70% MDPC-23 ($p<0.05$) and 80% OD-21 ($p<0.05$) were found apoptotic (Figure 2.2). Macrophages seemed to be more prone to partially polymerized Single Bond-induced apoptosis, since nearly 90% of the cells were found to be apoptotic at both time periods ($p<0.001$) (Figure 2.2). We observed that the process of pulp cell and macrophage death induced by an adhesive resin is characterized by typical morphological changes of apoptosis, such as nuclear condensation, rounding, and detachment of the cells (Figure 2.3).

Since the condition of partial polymerization seems the most relevant to the clinical use of an adhesive resin in deep cavity preparations, with or without pulp exposure, we decided to evaluate this condition in more detail with a time course experiment. Macrophages were again the cells that were demonstrated to be more sensitive ($p<0.05$) to partially polymerized Single Bond induced apoptosis (Figure 2.4). We observed a significant number of apoptotic macrophages after a 6-hour exposure, and by 12 hours most cells were apoptotic. In contrast, MDPC-23 presented a significant number of apoptotic cells after 9 hours ($p<0.05$) (Figure 2.5) and OD-21only after 12 hours ($p<0.05$) (Figure 2.6) as compared to untreated controls (Figure 2.4). We show here photomicrographs of a time course experiment with MDPC-23 as a demonstration that the morphological changes indicative of apoptosis are temporally correlated with the patterns of apoptosis depicted by the flow cytometric analyses (Figure 2.4) The

morphological changes observed in these cells are closely correlated with the patterns of apoptosis depicted through the flow cytometric analysis (Figure 2.5 and 2.6)

Adhesive Resin Affects the Cell Cycle of Pulp Cells and Macrophages

To study the effect of an adhesive resin at several degrees of polymerization on pulp cell and macrophage cell cycle, we performed a similar experiment as described above and performed cell cycle analysis. The three cell lines showed a normal cell cycle pattern while not exposed to the Single Bond (Figure 2.7). They presented few apoptotic cells, and the majority of the cells were in G_1 phase of cell cycle. In contrast, a dramatic shift in cell cycle profile was observed after a 12-hour exposure of every cell studied here to unpolymerized Single Bond (Figure 2.7). Under this condition, essentially all cells escaped from cell cycle and were now grouped as a sub-G_0/G_1 population. When cells were exposed to partially polymerized Single Bond, the effect on cell cycle was not as intense as the one observed with unpolymerized Single Bond, but followed a similar trend (Figure 2.7). The proportion of cells at sub-G_0/G_1 was increased as compared to untreated controls, and this was concomitant with a smaller the proportion of cells in the cell cycle (Figure 2.7). Macrophages were the cells that were more sensitive to the cytotoxic effect of the adhesive resin, since approximately 90% of the cells were in sub-G_0/G_1 phase, as compared to 45% MDPC-23, and 50% OD-21. The exposure to polymerized Single Bond essentially did not affect the cell cycle patterns of MDPC-23 and OD-21, as compared to controls (Figure 2.7). In contrast, macrophages exposed to polymerized Single Bond showed more sub-G_0/G_1 cells compared to controls, and a

decrease in the proportion of cells in S phase that was accompanied by a G2 cell cycle arrest (Figure 2.7) The trends of cell cycle profile observed at 24 hours were similar to the ones observed at 12 hours, but the cytotoxic effects were more pronounced (Figure 2.8).

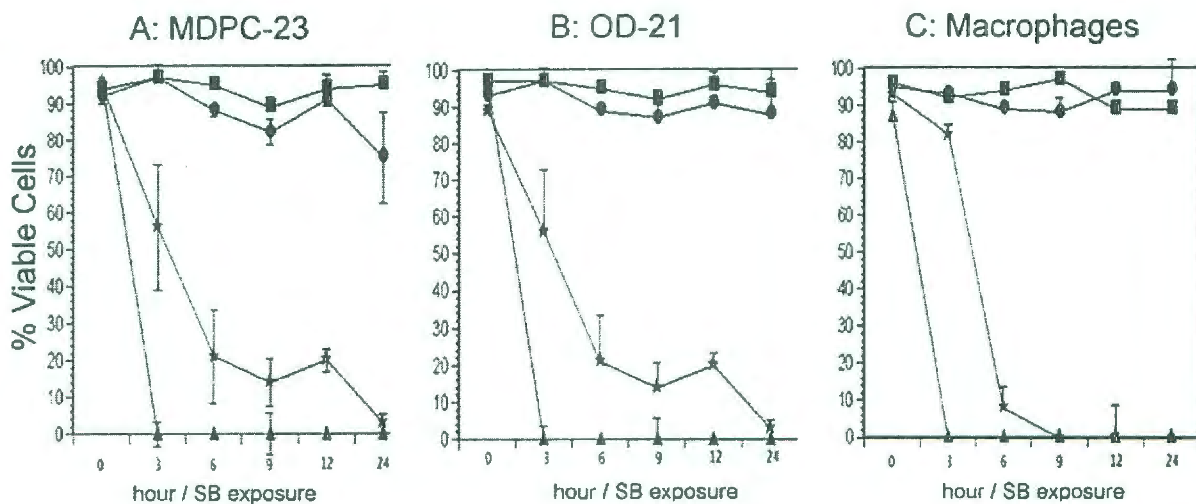
We performed a time course experiment to examine in more detail the changes in cell cycle profiles induced by a partially polymerized adhesive resin. These experiments demonstrated that partially polymerized Single Bond induced an increase in the proportion of sub-G_0/G_1 MDPC-23 after 9 hours (Figure 2.9). At 12 hours, a transient phase of G_2 cell cycle arrest was observed (Figure 2.9). Both, at 12 and 24 hours exposure times to partially polymerized Single Bond, the proportion of MDPC-23 cells in S phase was reduced in comparison to controls (Figure 2.9). Taken together, these data suggests that the MDPC-23 exposed to partially polymerized Single Bond were either undergoing apoptosis or were quiescent, non-proliferating cells. A similar trend was observed with OD-21, (Figure 2.10) but these cells started to show changes in cell cycle at 9 hours and at 24 hours the proportion of cells in sub-G_0/G_1 was increased as compared to MDPC-23. Among the three cell types evaluated, macrophages were the ones that showed the most significant changes in cell cycle upon exposure to partially polymerized Single Bond (Figure 2.11). After a 12 or 24 hour-exposure to partially polymerized Single Bond, the majority of the cells were in sub-G_0/G_1. Untreated MDPC-23, OD-21, and macrophages presented fairly constant cell cycle profiles during the time of the experiments (Figures 2.9, 2.10, 2.11).

Percentages of MDPC-23, OD-21 and macrophages at each cell cycle phase (G1, S and G2) are depicted at (Figure 2.12). A G2 cell cycle arrest at 12 hours was observed

on MDPC-23, OD-21 at 12 hours ($p<0.05$), whereas macrophages showed a G2 cell cycle arrest that was not statistically significant different as compared to untreated. A similar trend, with a G2 cell cycle arrest was observed at 24 hours in OD-21 and macrophages group but not in MDPC-23 ($p>0.05$). Taken together, these findings suggest that cells exposed to partially polymerized Single Bond were either undergoing apoptosis or were quiescent, non-proliferating cells; which is in consensus with decreased cell proportion in S phase at 12 and 24 hours ($p>0.05$)(Figure 2.12)

HEMA did not Affect Cell Viability or Morphology

We decided to evaluate HEMA's effect on viability of MDPC-23, OD-21 and macrophages, because this is a low-molecular weight hydrophilic monomer that is frequently present in adhesive resin systems. We observed that exposure of these cells to 10-1000 nM HEMA for 24 hours did not have a significant effect on the viability of the cells evaluated (Table 2.1), nor in cell morphology (Figure 2.13)



Symbols:

■ Untreated,
▲ Unpolymerized,
★ Partially Polymerized
● Polymerized

Figure 2.1. Time course experiment to study effect of degree of polymerization of an adhesive resin (Single Bond) in dental pulp cell viability: A) Odontoblast- like cells (MDPC-23), B) Undifferentiated pulp cells (OD-21) and C) Raw MØ were exposed to Single Bond (3M). Unpolymerized, (0 s) or partially polymerized (10 s) conditions induce cell death but its polymerized condition (40 s) did not cause significant cell death. Trypan Blue exclusion assay was used to detect necrotic cells.

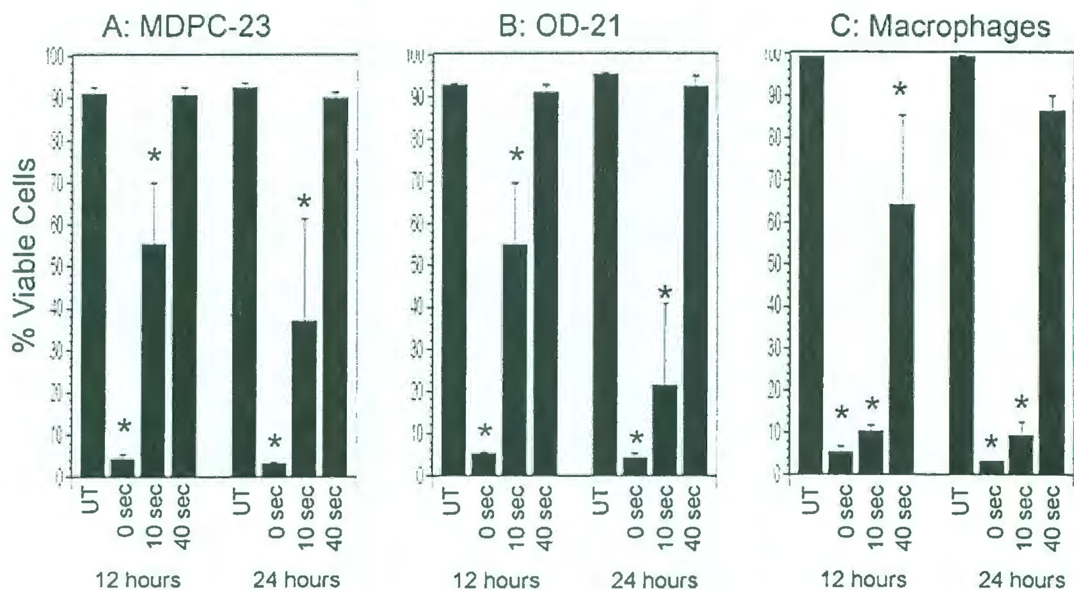


Figure 2.2. Unpolymerized and partially polymerized Single Bond treated groups showed a strong induction of cell apoptosis at 12 or 24 hours. A) Odontoblast- like cells (MDPC-23), B) Undifferentiated pulp cells (OD-21) and C) Raw MØ were exposed to Single Bond (3M). Single Bond in its polymerized condition did not shown significant cell death. Apoptotic cells were identified with propidium iodide in the treated groups by flow cytometric analysis. *Statistical significant difference between untreated groups and different concentrations are indicated with asterisk

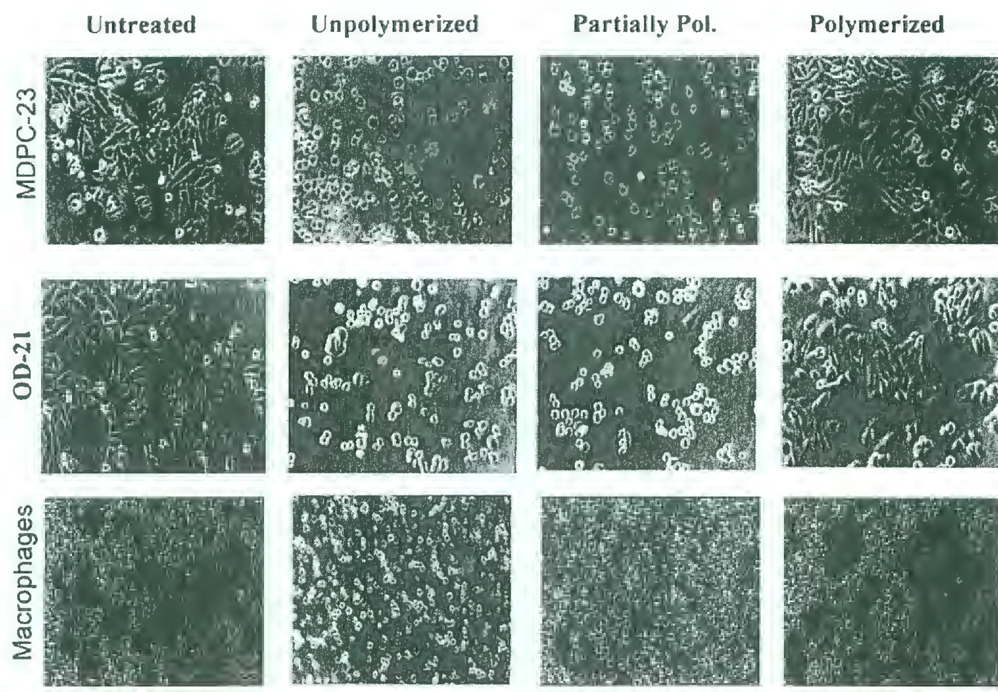


Figure 2.3: Photomicrographs at 100X of MDPC-23, OD-21 and Macrophages showing typical morphological changes in apoptotic cells. Adhesive resin (SB) unpolymerized (0 s) or partially polymerized (10 s) induced apoptosis of dental pulp cells and Raw macrophages. In contrast the polymerized (40 s) SB did not show cell morphology changes.

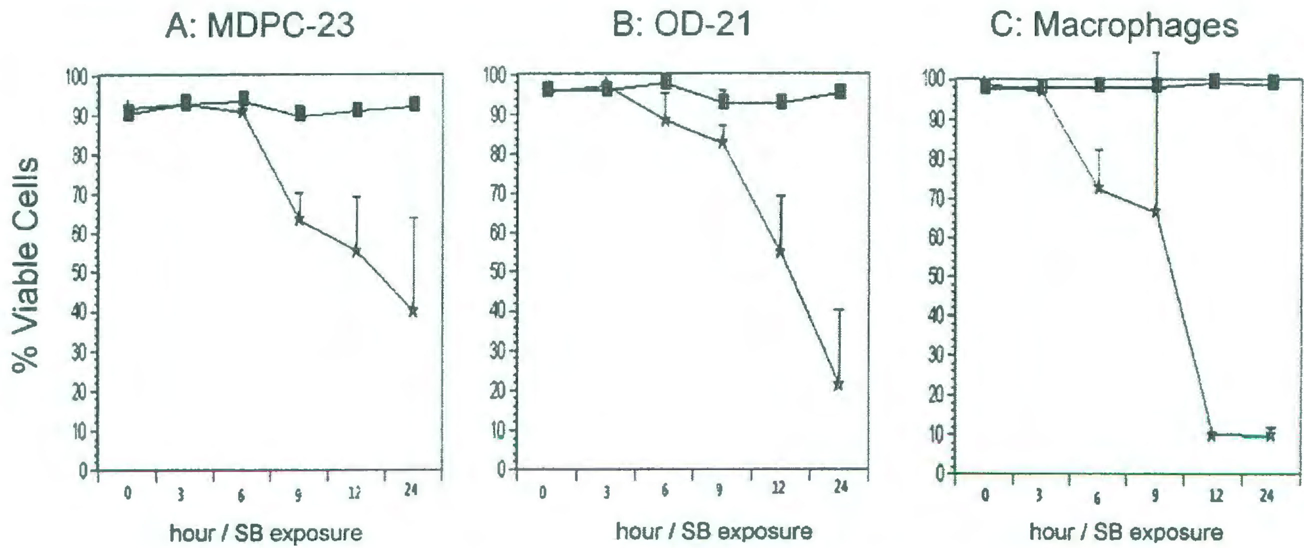


Figure 2.4: Time course experiment showing partially polymerized SB induced apoptosis of dental pulp cells andRaw macrophages. A) Odontoblast- like cells (MDPC-23), B) Undifferentiated pulp cells (OD-21), C) Raw MØ were exposed to partially polymerized (10 s) adhesive resin (Single Bond, 3M) trypan blue exclusion assay was used to measure integrity of cell membrane and to identify necrotic cells.

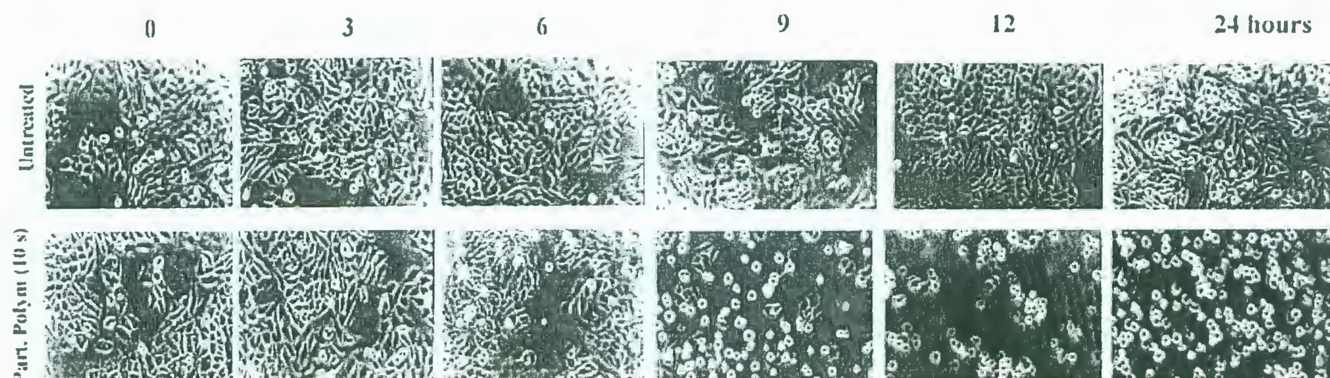


Figure 2.5 Photomicrographs at 100X, of (MDPC-23) showed cell apoptosis patterns at 9, 12 and 24 hours on cells treated with partially polymerized adhesive resin (Single Bond, 3M).

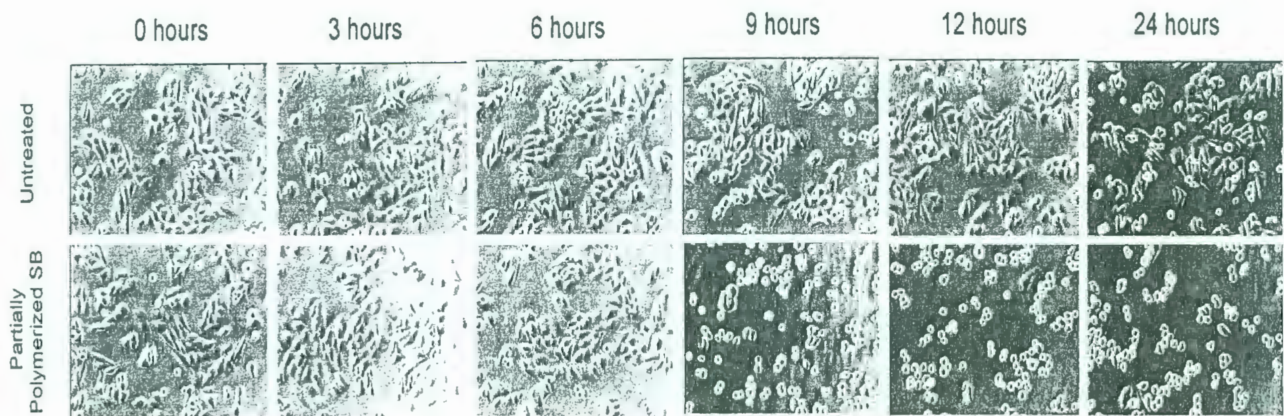


Figure 2.6. Photomicrographs at 100X, of (OD-21) shows cell apoptosis patterns at 9, 12 and 24 hours on cells treated with partially polymerized adhesive resin (Single Bond, 3M)

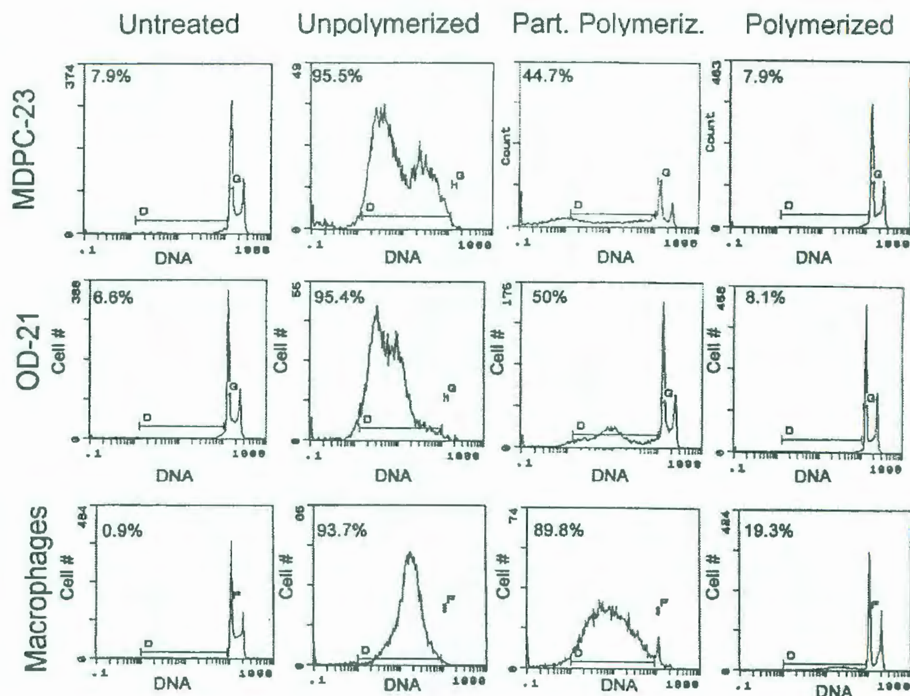


Figure 2.7. Flow cytometric analyses of Odontoblast- like cells (MDPC-23), Undifferentiated pulp cells (OD-21) and Raw MØ. Data shows that an unpolymerized (0 s) or partially polymerized (10 s) adhesive resin (SB) induced cell apoptosis after 12 hours, except on its polymerized (40 s) condition. Propidium Iodide was used to identify apoptotic cells. "D" corresponds to the fraction of apoptotic cells, which is depicted at the upper left corner of each graph

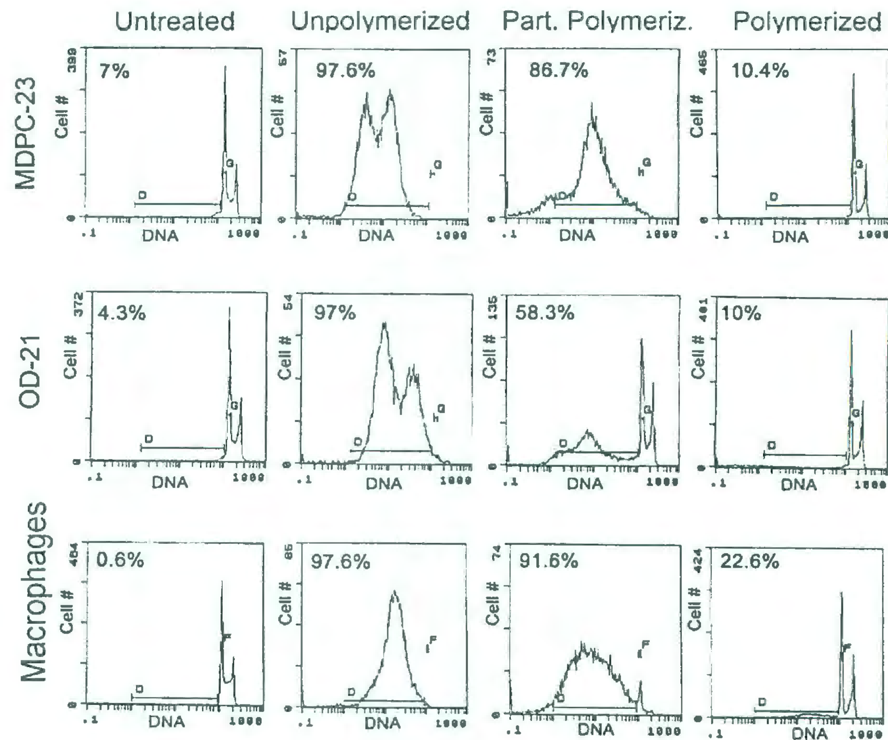


Figure 2.8. Flow cytometric analyses of Odontoblast- like cells (MDPC-23), Undifferentiated pulp cells (OD-21) and Raw MØ. Data shows that an unpolymerized (0 s) or partially polymerized (10 s) adhesive resin (SB) induced more pronounced cell apoptosis after 24 hours, but not at its polymerized (40 s) condition. Propidium Iodide was used to identify apoptotic cells. "D" corresponds to the fraction of apoptotic cells, which is depicted at the upper left corner of each graph.

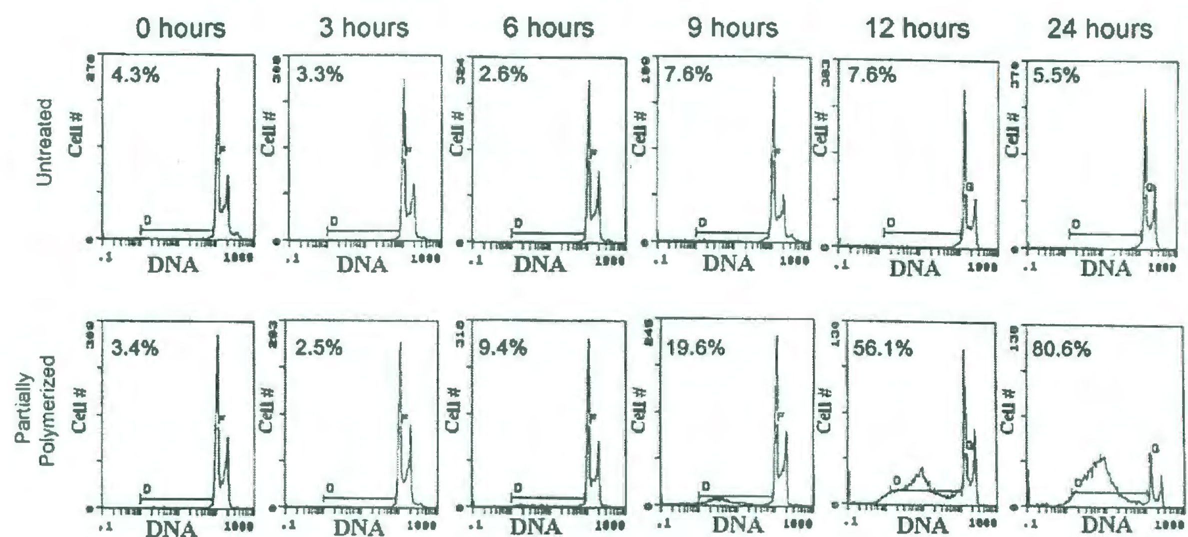


Figure 2.9. Time course exposure of (MDPC-23) to partially polymerized adhesive resin (10 s) (SB, 3M) shows that Single Bond started to induce apoptosis at 9 hours. Flow Cytometry with propidium iodide was used to identify apoptotic cells. "D" corresponds to the fraction of apoptotic cells, which is depicted at the upper left corner of each graph.

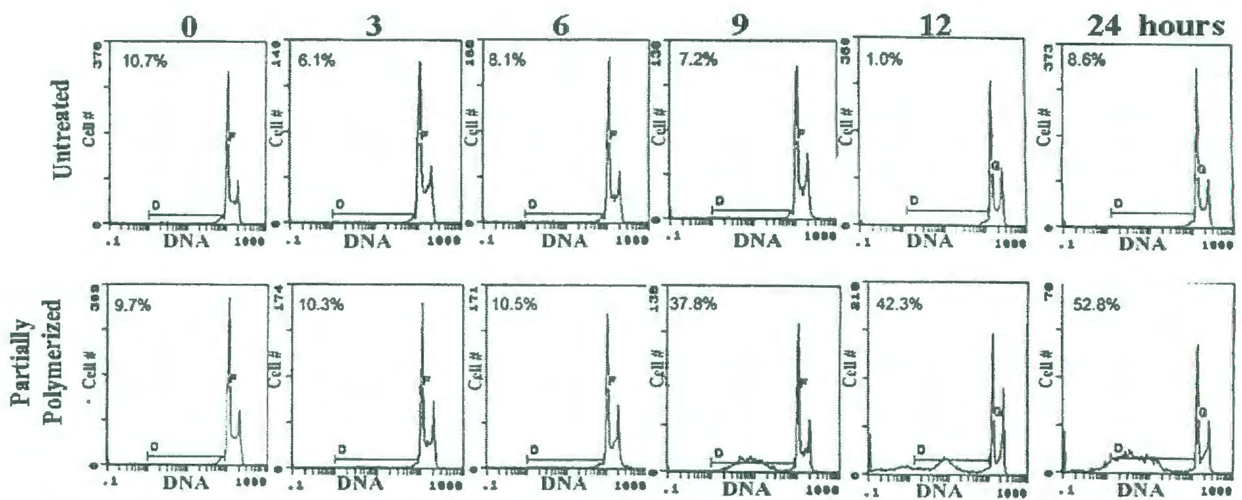


Figure 2.10. Time course exposure of (OD-21) to partially polymerized adhesive resin (10 s) (SB, 3M) shows that Single Bond started to induce apoptosis of Odontoblast-like cell at 9 hours. Flow Cytometry with propidium iodide was used to identify apoptotic cells. "D" corresponds to the fraction of apoptotic cells, which is depicted at the upper left corner of each graph

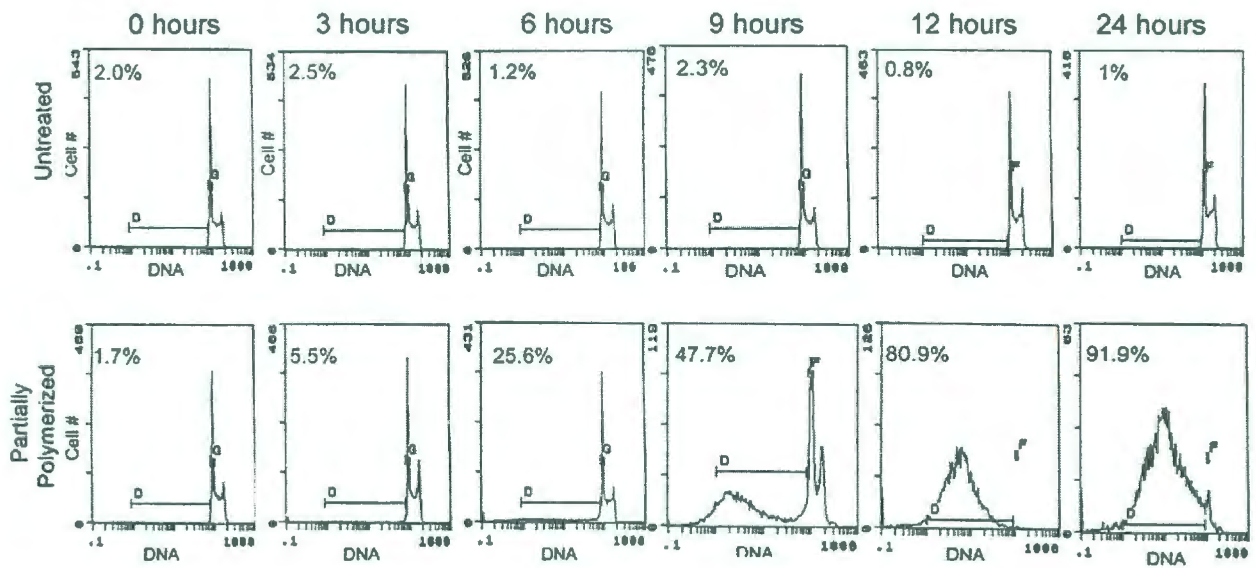


Figure 2.11. Time course exposure (Raw-MØ) to partially polymerized adhesive resin (10 s) (SB, 3M) shows that Single Bond started to induce apoptosis of undifferentiated pulp cells at 6 hours. Flow Cytometry with Propidium Iodide was used to identify apoptotic cells.

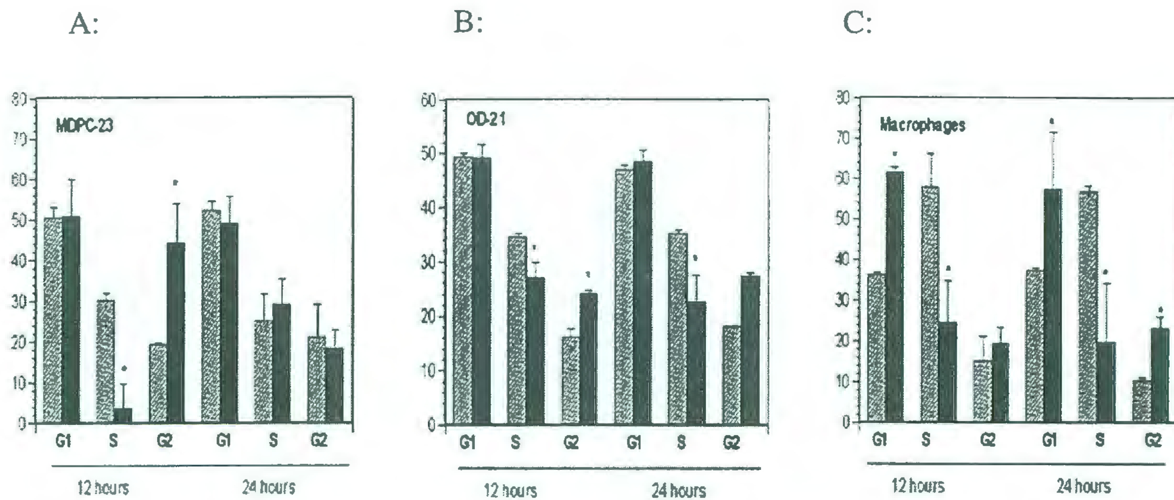


Figure 2.12. Percentage of MDPC-23, OD-21, and Macrophages at each phase of cell cycle (G1, S and G2) Gray bars depict untreated cells and solid black bars depict cells treated with partially polymerized Single Bond. A: MDPC-23, B: OD-21, and C: Raw MØ. *Statistical significant difference ($p \leq 0.05$) between untreated and Single Bond-exposed cells are indicated with an asterisk.

■ Untreated

❑ Partially Polymerized

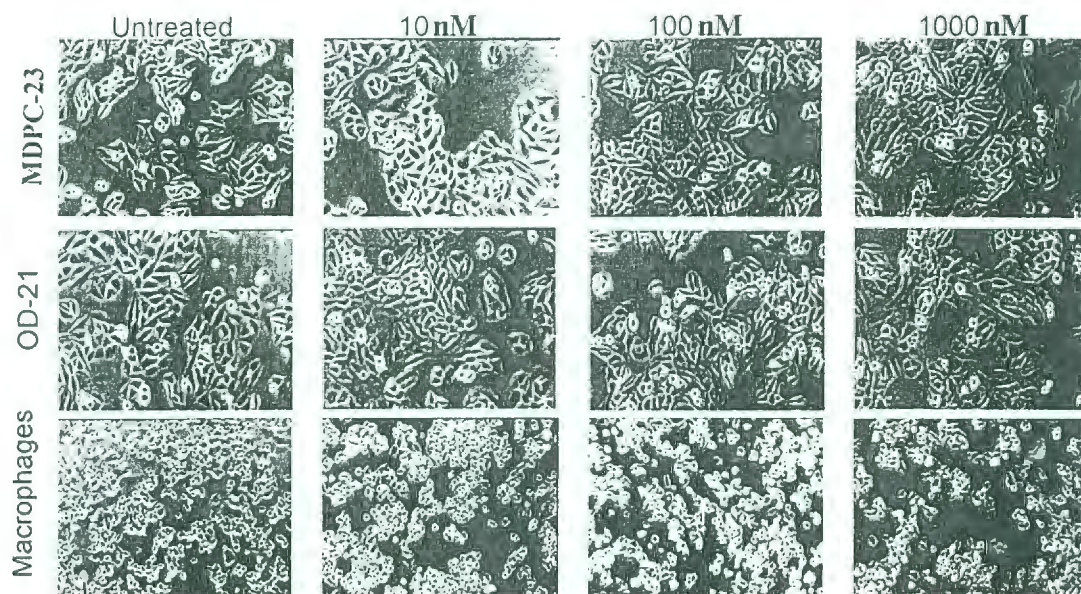


Figure 2.13. Photomicrographs at 100X showing that HEMA even at its highest concentration did not show cell morphology changes in MDPC-23, OD-21 or Macrophages exposed to 0 -1000 nM HEMA.

	Untreated	10 nM	100 nM	1000 nM
MDPC-23	98.80 (1.5)	97.90 (0.9)	97.60 (0.3)	98.30 (0.1)
OD-21	97.41 (1.5)	97.01 (0.5)	98.03 (5.1)	96.70 (2.12)
Macrophages	97.50 (0.4)	97.00 (1.5)	97.70 (0.4)	93.15 (2.9)

Table 2.1. Percentage of viable cells after exposure to HEMA concentrations. MDPC-23: Odontoblast-like cells, OD-21 Undifferentiated pulp cells, Raw Mø macrophages were exposed to Hydroxyethyl-methacrylate (HEMA) for 24 hours. Data is presented as percentage (%) of viable cells ± Standard deviations (in parentheses), as measured by trypan blue exclusion.

Discussion

The use of adhesive resins has increased significantly over the last years as a consequence of the increase in the indication of composite resin restorations. The apparent lack of cytotoxicity of these agents led to the suggestion that they could be utilized for direct pulp capping procedures (Cox, 1998; Kanca, 1990; Murray, 2000). Some *in vivo* studies have shown pulp repair and dentin bridging when adhesive resins were applied on micro-mechanical pulp exposures in non-human primate teeth, (Akimoto *et al.,* 1998; Kitasako *et al.,* 1999; Onoe *et al.,* 1995); (Costa *et al.,* 1999). On the contrary, the histological evaluation of human dental pulps treated directly with bonding agents has demonstrated inflammatory responses and lack of dentin bridge development (Costa *et al.,* 2000; Hebling *et al.,* 1999; Pereira *et al.,* 2000). Here we report that an adhesive resin can interrupt dentin repair by causing apoptosis of pulp cells that are involved in the process of induction of dentin regeneration (i.e. odontoblasts), apoptosis of cells that might replace dead odontoblasts (i.e. undifferentiated pulp cells), and apoptosis of key orchestrators of wound healing responses (i.e. macrophages). Furthermore, we report significant changes in cell cycle profiles upon exposure to unpolymerized or partially polymerized adhesive resin that results in cells that are either quiescent or dying.

The cytotoxic effects of resin monomers have been reported earlier in the literature (Hanks *et al.,* 1991; Ratanasathien *et al.,* 1995). The concentration of substances diffusing across the dentin into the pulp depends in part on the rate of removal of the toxins via pulpal circulation (Pashley, 1979). An adhesive resin releases initially greater amounts of its components, but this release tends to decrease over time (S.

Bouillaguet *et al.,* 1996, 1998; Ferracane 1990, 1994; Hanks *et al.,* 1992; Gerzina and Hume, 1994). This observation suggested that the higher risk of toxicity would happen immediately after placement of the composite material. The presence of unpolymerized monomers might enhance cytotoxicity by diffusion through dentinal tubules due to the small molecular size of them. Rathburn *et al.,* 1991, showed that the toxicity was decreased by 90% following removal of unpolymerized monomers from resin composites with organic solvents. Hanks *et al.,* 1992, reported that when monolayer cultures of Balb/c3T3 cells were exposed to HEMA, it caused irreversible inhibitory metabolic changes for all four parameters tested: DNA synthesis, protein synthesis, succinyl dehydrogenase activity, and total protein content. Gwinnett and Tay (1998) reported an irreversible injury to odontoblast cells when bonding agents were applied closest to the site of exposed cavity preparation.

We observed that soon after exposure to unpolymerized, or partially polymerized Single Bond, all cell types evaluated showed a process of nuclear condensation, rounding and detachment from the culture surface. This phenomenon happened through the process of apoptosis, in which cells commit suicide by activating caspases and downstream DNAses (Thompson, 1995). In agreement with Ferracane (1994) our results also indicated that the degree of polymerization of the adhesive resin is an important factor in the toxicity of the material. An inverse relationship exists between the degree of light-curing and adhesive resin cytotoxicity. Polymerized adhesive resin discs showed no toxic responses in our study, perhaps because less toxic elements are leached from polymerized resins. However, Gerzina and Hume (1995, 1996) detected the presence of TEGMA and HEMA released from resin composite material across dentin as late as 100 days

following polymerization. This might be explained by degradation with time and leaching from tags, and diffusion towards the pulp. Ferracane *et al.,* 1994, showed that even in fully set restorative materials substantial amount of short-chain polymers remain unbound, with the result that there is possible elution of leachable toxic components toward the pulp. Interestingly, Quinlan *et al.*, (2002), reported apoptosis of transformed human endothelial cell (ECV-304) when exposed to discs of the composite resin Spectrum or compomer Dyract light-cured for 40 s. This might be explained by increased sensitivity of endothelial cells to composite resin-induced cytotoxicity, as well as differences in study methodologies. In agreement with our study, partially polymerized composite resin (1 s and 4 s) samples caused stronger induction of apoptosis than polymerized specimens. (Quinlan, 2002)

Previous studies have shown that a wet environment interferes with the polymerization of resinous materials (Spahl *et al.*, 1999 Geurtsen *et al.*, 1998). Since humidity inhibits complete polymerization of adhesive resins, an increase in the diffusion of hydrophilic monomers towards the pulp might be observed (Rueggeberg and Margeson, 1990). In sites of pulp exposure, increased oxygen tension caused by the presence of hemorrhagic red blood cells might further inhibit the polymerization of adhesive resins. Therefore, we used unpolished adhesive resin discs to simulate an oxygen-inhibited layer in all our specimens.

Pulp therapy with adhesive resins allows for continuous release of unreacted resin components, which might be responsible for the cell death in many of the *in vivo* studies (Hebling *et al.,* 1999; Costa *et al.*, 2000; Pereira *et al.,* 2000; Schmaltz *et al.,* 2002). Previous studies have shown that in clinical situations where there is still more than 0.5

mm of remaining dentin thickness the cytotoxicity of the resin monomers is decreased by up to 50%, (Hanks *et al.,* 1988, 1989; Meryon & Brook, 1989). Furthermore when more than 0.5 mm dentin thickness is left, dentin adsorbs substances in the tubules and further limits the diffusion of these substances (Hanks *et al.,* 1994). In the present *in vitro* study we simulated the direct pulp capping procedure with adhesive resins at the pulp exposure site. Based on our results, we speculate that the adhesive resin causes intense pulp damage under these circumstances *in vivo.* We have shown that the cytotoxic effect manifests at least in part, through induction of apoptosis of odontoblasts, undifferentiated pulp cells, and macrophages present at the site of exposure.

Many *in vitro* studies have reported that adhesive resin components are cytotoxic to metabolic activity when applied to fibroblasts (Hanks 1992; Costa *et al.,* 1999). Costa *et al.,* in 1999 evaluated the cytotoxic effect of Single Bond among other one bottle adhesive resins, showing that it was highly cytopathic to MDPC-23. Also cytotoxic effects of resinous materials and their components, such as HEMA, have been reported (Luster, 1989). Since unpolymerized and partially polymerized experimental conditions induced significant cell death which was predominantly caused by the adhesive resin, we decided to study the mode of cell death, *i.e* programmed cell death ("apoptosis") or by "accidental" cell death, ("necrosis"). We also evaluated if there were any changes in cell cycle of experimental cells as compared to untreated controls. We observed that unpolymerized and partially polymerized adhesive resin induces a quick and pro-apoptotic activity. We also observed a change in cell cycle of cultures of odontoblast-like cells (MDPC-23), undifferentiated pulp cells (OD-21) and Raw macrophages that are compatible with cytotoxic cellular responses. In contrast, when cells were exposed to

fully cured adhesive resin, cytotoxicity was reduced. We did not observe significant changes in the viability of treated MDPC-23 and OD-21 as compared to controls, but it did affect cell viability of macrophages. This emphasizes the relevance of the degree of polymerization to the cytotoxicity of adhesive resins.

Several authors have reported that direct pulp capping procedures with adhesive resins do not induce dentin bridge formation at the site of pulp exposure (Gwinnett and Tay, 1998; Hebling *et al.*, 1999). *In vitro* we observed apoptosis between 80- 100 % of odontoblasts, undifferentiated stromal cells and macrophages as a result of exposure of adhesive resin in its unpolymerized (0 s) and partially polymerized condition (10 s). Odontoblasts are terminally differentiated cells that do not proliferate *in vivo*, and undifferentiated pulp cells are believed to be responsible for the replacement of dead odontoblasts. If odontoblast and undifferentiated stromal cells become apoptotic the process of dentin-bridge formation will be hindered.

Macrophages, which are important mediators in tissue organization and wound healing, were also found apoptotic upon exposure to Single Bond. Therefore the pulp tissue might be even further compromised in its capacity to organize a healing process.

Requirement of minimum number of cell cycles prior to withdrawal from cell cycle before inductive signaling for differentiation implies that a certain level of competence is required before the cell can respond. Such cell competence would be expected to be especially important in reparative situations in mature tissues and must be more fully understood in terms of recruitment of new odontoblast-like cells (Tziafas, 2000). In addition to the direct induction of apoptosis, we also observed changes in cell cycle pattern that can be translated into reduction in the cell proliferation rate. This means

additional evidence to explain the observed lack of dentin bridge formation *in vivo* studies, since cell proliferation is a requirement for any healing process throughout the organism. We also speculate that cell cycle alterations may be responsible for not having pulp cell proliferation, and dentin regeneration when adhesive resins are applied directly over a pulp exposure site.

In summary, we have shown here that adhesive resin induces apoptosis or cell cycle arrest of cells that are major players in the regeneration of dentin. This might explain, at least in part, the lack of dentin bridge formation in teeth treated with direct pulp capping with adhesive resins, and the disorganization observed in the odontoblastic layer of teeth with deep carious lesions that were restored with adhesive resins and composite. Therefore, this study supports the recommendation of the manufacturer (3M) that does not indicate the use of Single Bond for direct pulp capping. We believe that the understanding of the mechanisms of cytotoxic responses induced by restorative materials will provide the clinicians with information that can be used when deciding to restore deeply carious lesions or pulp exposures with composite resins.

Conclusions

Under the conditions of this study:

1) Unpolymerized or partially polymerized Single Bond induces apoptosis of odontoblast-like cells, undifferentiated pulp cells, and macrophages.

2) Unpolymerized or partially polymerized Single Bond induces escape from cell cycle or cell cycle arrest of odontoblast-like cells, undifferentiated pulp cells, and macrophages.

3) The degree of polymerization of the adhesive resin plays a significant role in the intensity of the cytotoxic response of dental pulp cells and macrophages.

4) The cytotoxicity of an adhesive resin evaluated in this *in vitro* study suggests its contra-indication for direct pulp capping procedures.

5) Based upon our findings in this study, it is recommended to polymerize more than 10 seconds adhesive resins when those will be placed in deep dentin.

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Chapter III

AN ADHESIVE RESIN AND THE HYDROPHILIC MONOMER HEMA INDUCE VEGF EXPRESSION ON DENTAL PULP CELLS AND MACROPHAGES

Abstract

Angiogenesis is the fundamental process by which new blood vessels are formed from pre-existing capillaries. Vascular endothelial growth factor (VEGF) plays an important role in orchestrating responses by inducing endothelial cell proliferation, migration and survival. The purpose of this *in vitro* study was to examine if VEGF is upregulated in dental pulp cells exposed to an adhesive resin or to HEMA (2-hydroxyethyl methacrylate). Mouse odontoblast-like cells (MDPC-23), mouse-undifferentiated pulp cells (OD-21), fibroblasts and Raw Macrophages (Mø), were cultured in triplicate wells with DMEM/10% FBS. After 24 h, cells were exposed to 0, 10, 100 or 1000 nM HEMA. Alternatively, cells were exposed to discs weighing 11 (± 1) mg the adhesive resin Single Bond (3M) at 3 conditions of polymerization: 0, 10 or 40 s light-curing time. The adhesive resin was placed over 0.4 μm pore size polycarbonate transwell inserts. After 24 h, ELISA was performed in conditioned medium for mouse VEGF (Quantikine, R & D systems). T-test or one-way ANOVA were used for statistical analyses. We found that VEGF was upregulated in MDPC-23 exposed to HEMA ($p<0.001$) and to Single Bond ($p<0.018$). VEGF was also upregulated in Mø exposed to

HEMA ($p<0.001$) or Single Bond ($p=0.001$). In contrast, VEGF expression was not upregulated in undifferentiated pulp cells (OD-21) or fibroblasts exposed to either HEMA or Single Bond ($p>0.05$). These results demonstrate that an adhesive resin or HEMA induces expression of VEGF by macrophages and odontoblasts, but not by undifferentiated pulp cells or fibroblasts.

Introduction

Vascular endothelial growth factor (VEGF) plays a fundamental role during development, wound healing, and for the maintenance of life. Its importance is underlined by the observation that VEGF knockouts die before birth (Ferrara *et al.*, 1996) VEGF is produced by several cell types, such as keratinocytes, macrophages, mast cells and fibroblasts. VEGF was also shown to increase vascular permeability and to be involved in the occurrence and progression of inflammation (Dvorak, 1995).

During embryogenesis, VEGF is essential for differentiation of "de novo" blood vessels in a process called "vasculogenesis". VEGF is also involved in the process of "angiogenesis" which is the sprouting of new capillaries from preexisting vessels. The latter term was first used in 1935 to describe the formation of new blood vessels from pre-existing capillaries in the placenta (Hertig, 1935)· In adults, angiogenesis is a critical component of wound healing, as well as in inflammatory responses. Disruptions in the regulation of the angiogenic response have been correlated with delayed healing of wounds (Di Pietro *et al.,* 1996). Angiogenesis per se can enhance the severity of the inflammatory process due to the increased transport of inflammatory cell nutrients and oxygen to the site of inflammation (Johnson, 1999).

Several recent manuscripts address the potential role of VEGF in the biology of the dentin-pulp complex. VEGF among other angiogenic factors was found to be expressed in dentin matrix. Its expression in dentin suggests a contribution to the overall reparative response of the dentine-pulp complex (Roberts-Clark and Smith, 2000). Artese *et al.,* 2002 reported that VEGF expression in stromal cells in healthy pulps was strongly positive in the inflammatory infiltrate in irreversible pulpitis. In contrast, it was found to

be slightly, but significantly decreased in the stromal cells in irreversible pulpitis. VEGF ranged from 20-100% and irreversible pulpitis ranged from 0-100%. The microvessel density in healthy pulps was higher as compared to those with irreversible pulpitis. It is known that adhesive resins or calcium hydroxide induce pulp-vascularization. Neovascularization is regulated by several molecules, which can either have a positive or a negative regulatory effect (Folkman and Klagsbrun, 1987). The nature and origin of angiogenic stimuli in pulp tissue are still uncertain. The aim of this study is to examine if vascular endothelial growth factor (VEGF) is upregulated in pulp cells exposed to an adhesive resin or HEMA *in vitro*, and to determine which cells synthesize this factor.

Materials Methods

An *in vitro* system was designed to study VEGF expression levels induced by exposure to 2-hydroxyethyl-methacrylate (HEMA) or an adhesive resin. Mouse odontoblast-like cells (MDPC-23), and undifferentiated mouse pulp cells (OD-21), (gift from C.T. Hanks), as well as mouse fibroblasts or mouse macrophages (Raw, ATCC) were cultured at 37°C in a humidified CO_2 incubator. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 250 μg/ml L-glutamine, 125 units/ml Penicillin, and 125 μg/ml Streptomycin (all from Gibco BRL, Grand Island, NY, USA). Cells were plated in 12-well-plates, in a concentration of 6 x 10^4 cells/well for MDPC-23 and OD-21, 8 x 10^4 cells/well for fibroblasts and 5 x 10^4 cells/well for macrophages. Zero, 10, 100, or 1000 nM of HEMA (batch # 868-77-9, Aldrich Chemical Company, St. Louis, MO) was added to the culture medium in triplicate wells per condition. Experiments using the adhesive resin, Single Bond (3M, Minneapolis, MN), were performed by culturing the cells in 12-well plates and placing the specimens over permeable membrane inserts (0.4 μm pore size, Corning, Costar, Cambridge, MA) to prevent direct physical interaction between adhesive resins and cells.

To measure baseline expression of VEGF of each cell line, 4 x 10^4 cells/well were seeded in 12-well plates and the medium was changed after 24 hours. VEGF expression was quantified by ELISA from harvested medium.

Specimen Preparation

Single Bond discs measuring 5 mm x 2 mm were prepared under sterile conditions with a prefabricated mold. The specimens were polymerized with an Optilux 401 light-curing unit (Demetron, Kerr, Danbury, CT) calibrated at 800 mW/cm^2. Specimens were light-cured for 10 s, to obtain the partially polymerized group; and were light-cured for 40 s, to obtain the completely polymerized group. All specimens were weighed in a calibrated electric balance and only those weighing 11 ± 1 mg were utilized. Unpolymerized (0 s light-curing time) specimens (11 ± 1 mg) were used as positive control and untreated cells were used as negative controls.

Trypan Blue Exclusion Assays

Cell viability was assessed using trypan blue exclusion assay technique, which measures integrity of the cell membrane (Freshney, 1987). After a 0-24 h exposure to either Single Bond or HEMA, MDPC-23, OD-21, fibroblasts or macrophages were retrieved with a solution of 0.25% trypsin supplemented with 1 mM EDTA (Gibco). Aliquots of harvested cells from each well were mixed with 0.4 % trypan blue dye (1:1 by volume). The number of necrotic cells and the total number of cells was determined from triplicate wells for each condition by means of a hemacytometer with an optical microscope at 100x.

Enzyme-linked Immunosorbent Assay (ELISA)

The Quantikine Cytokine ELISA Kit (R&D Systems, Inc. Minneapolis, MN) was used to quantify mouse VEGF expression in the supernatants of MDPC-23, OD-21,

fibroblasts, and macrophages induced by exposure to either Single Bond, or HEMA. Cells were cultured for 24 hours as previously described and conditioned medium was collected and centrifuged for 5 min, at 4°C at 13,000 rpm. Conditioned medium (100 μl) was placed in each well of a 96-well plate, which was previously coated with monoclonal antibody for VEGF (Quantikine, RD Systems), and incubated for 2 hours at room temperature. Subsequent to aspirating and washing away any unbound substances, a secondary antibody was added to each well, and incubated for 2 additional hours. After aspirating and washing the wells, incubation with a chromogenic substrate solution was performed and after 30 minutes, the reaction was stopped by stop solution. Readings for optical densities were recorded with Spectrophotometer calibrated to 450 nM wavelenght (DU-20, Beckman, USA).

Statistical Analyses

The statistical analyses of the data were performed by Student's t-test and one-way ANOVA with SigmaStat 2.0 software (SPSS, Chicago, IL). Statistical significance was determined at $p \leq 0.05$.

Results

Effect of Single Bond on VEGF Expression

To examine how this angiogenic process is regulated by dental pulp cells, fibroblasts and macrophages, we first measured the baseline expression of VEGF by ELISA's from 100 μl of harvested medium at 24 h. We observed that odontoblast-like cells and undifferentiated pulp cells express higher baseline levels of VEGF as compared to fibroblasts and macrophages. For example, MDPC-23 expressed 256 (±4.8) pg/ml and OD-21 expressed 495 (± 46.5) pg/ml, whereas macrophages expressed 52.03 (±16.68) pg/ml and fibroblast expressed 175.5 (±49.3) pg/ml (Figure 3.1). Then, we evaluated how the four cell types respond to stimulation by an adhesive resin (Single Bond). We observed that the expression of VEGF was upregulated in odontoblast-like cells ($p=0.004$) and macrophages ($p<0.001$), but not in undifferentiated pulp cells and fibroblasts ($p>0.05$) (Figure 3.2)

In light of the results described above and taking into consideration the fact that HEMA is one of the most common components of adhesive resins, we exposed cells to 0-1000 nM HEMA and evaluated VEGF expression levels (Figure 3.3). We observed that the monomer HEMA is sufficient to upregulate VEGF expression in both odontoblast-like cells ($p<0.001$) and macrophages ($p<0.001$), but not in undifferentiated pulp cells ($p>0.05$) and fibroblasts ($p>0.05$) (Figure 3.3).

Effect of Polymerized Single Bond or HEMA on Cell Morphology

We also observed that the induction of VEGF expression mediated by Single Bond or HEMA was not accompanied by changes in cell morphology that could be detected at the light-microscopy level (Figure 3.4 and 3.5).

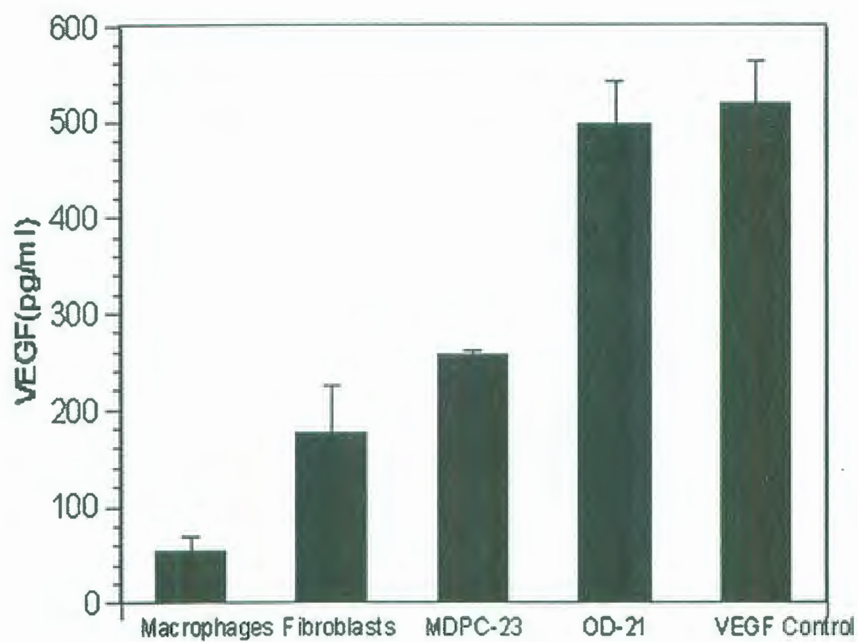

Figure 3.1. VEGF baseline expression of dental pulp cells and Raw macrophages seeded at 4 x 10^4cell density. ELISA was used to quantify VEGF expression on (MDPC-23, OD-21, fibroblasts and Raw Macrophages) Recombinant mouse VEGF was used as a control.

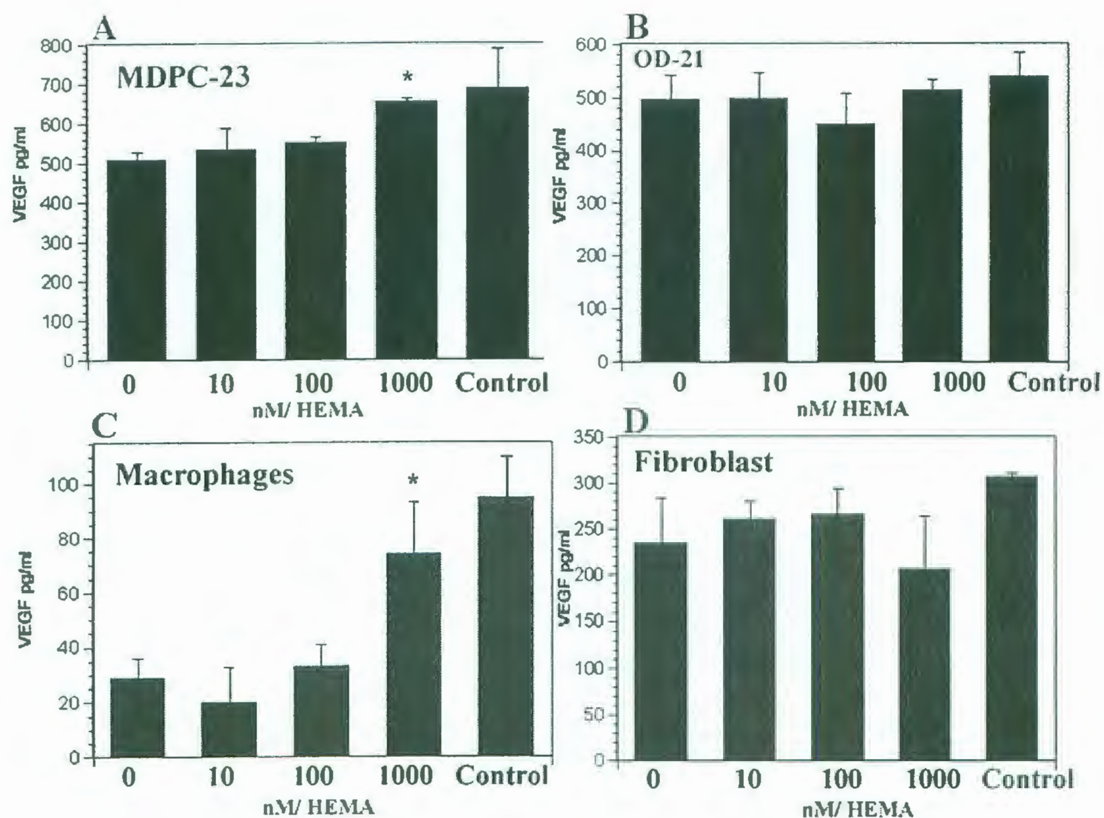

Figure 3.2. VEGF expression was upregulated in MDPC-23 or MØ exposed to 1000 nM HEMA but not by OD-21 or by fibroblasts, after 24 hours. A: (MDPC-23) B: (OD-21), C: (Raw Macrophages) and D: (fibroblasts) Recombinant mouse VEGF was used as a control. *Statistical significant difference between untreated groups and different concentrations are indicated with asterisk.

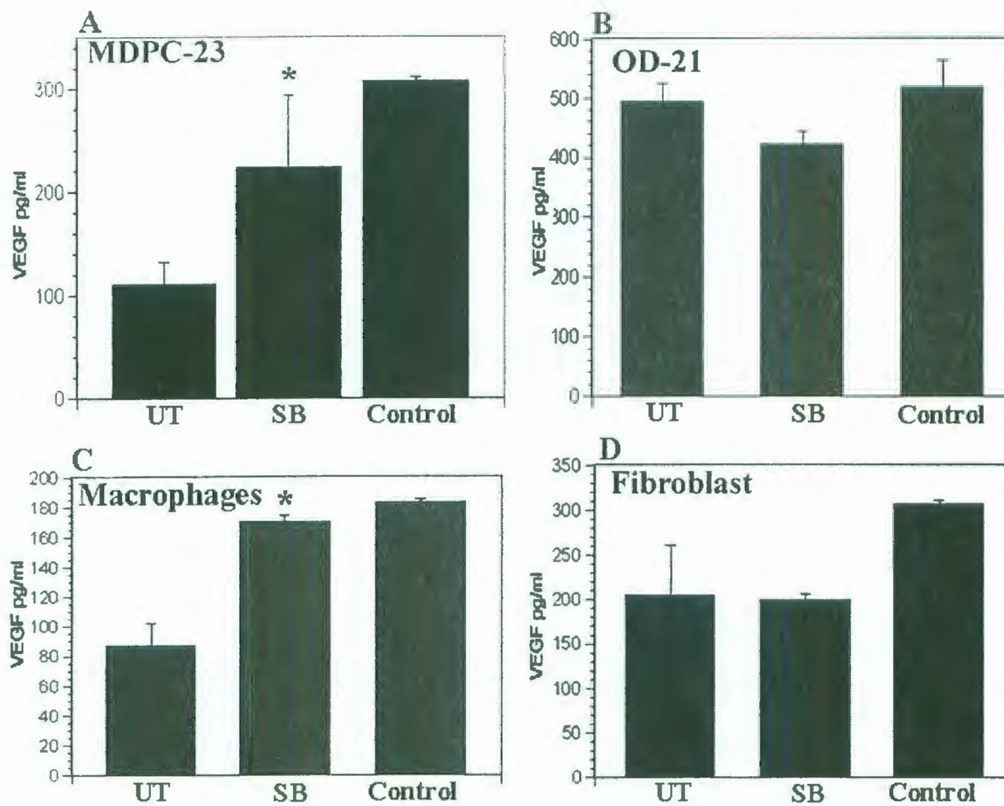

Figure 3.3. VEGF expression was upregulated in MDPC-23 (4x 10^4) or MØ (5 x10^4) exposed to SB (40 s) but not by OD-21 (4x 10^4) or by fibroblasts (8 x 10^4), after 24 hours. A: (MDPC-23) B: (OD-21), C: (Raw Macrophages) and D: (fibroblasts) Recombinant mouse VEGF was used as a control. *Statistical significant difference between untreated groups and different concentrations are indicated with asterisk.

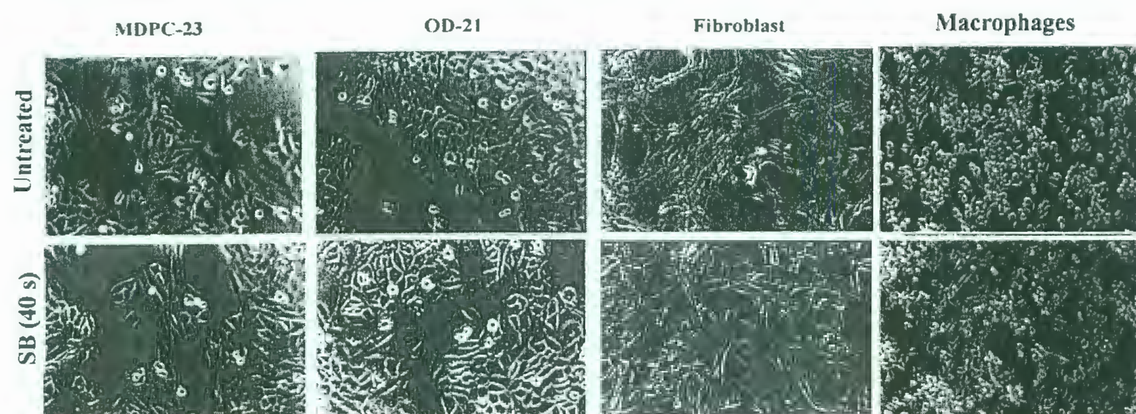

Figure 3.4. Photomicrographs at 100X of MDPC-23, OD-21, fibroblasts and macrophages showed that after 40 s of light-curing time, SB did not cause detectable cell morphology changes

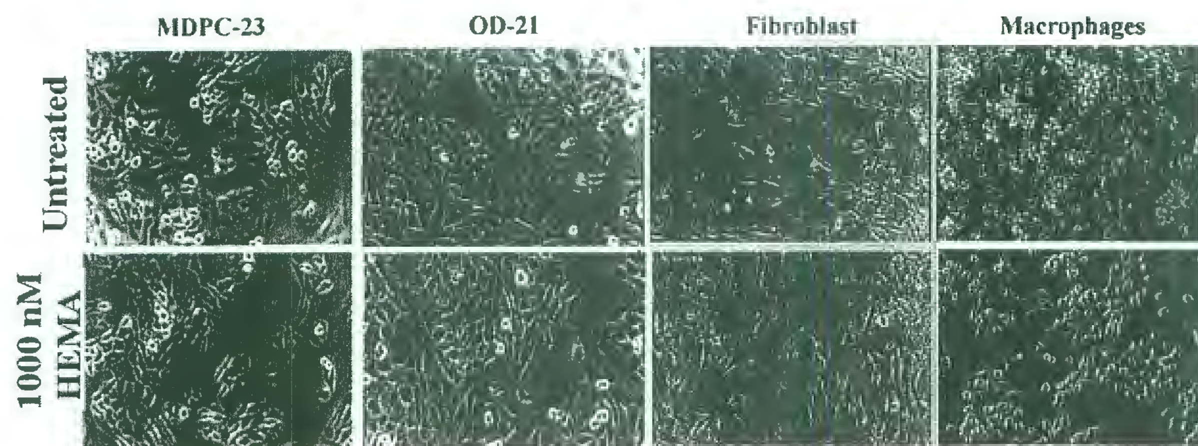

Figure 3.5. Photomicrographs at 100X of MDPC-23, OD-21, fibroblasts and macrophages showed that being exposed to 1000nM HEMA did not cause detectable cell morphology changes

Discussion

The direct application of an adhesive resin to sites of pulp exposure was shown to induce an increase in pulp vascularization (Costa *et al.*, 2000). The release of inflammatory mediators and cytokines can contribute to the wound healing process in the pulp or lead to generalized inflammatory reactions and pulp necrosis through increased vascular permeability and tissue pressure in the low-compliancy pulpo-dentinal environment (Heyeraas *et al.,* 1999). VEGF is an angiogenic growth factor that binds to specific surface receptors in endothelial cells and enhances tissue vascularization and vascular permeability.

In the present study, we observed high expression levels of VEGF in pulp cells. This finding suggests that this growth factor plays an important role in dental pulp vascularization. We observed an upregulated expression of VEGF in odontoblast-like cells (MDCP-23) and Raw macrophages when cells were exposed to Single Bond or HEMA. However VEGF was not upregulated in a precursor odontoblastic cell line (OD-21), or in fibroblasts. This suggests that the odontoblasts play an important role in the angiogenic response observed in healthy pulps. And macrophages may intensify angiogenesis when inflammation is present in the pulp tissue. Artese *et al.,* 2002, demonstrated that VEGF is expressed in healthy human dental pulp tissues. These authors analyzed pulp microvessel density and VEGF expression by immunostaining. This study demonstrated higher microvessel density in healthy pulps when compared to irreversible pulpitis. The downregulation in the irreversibly inflamed cells could be due to their presence in a low compliance space such as it is in the dental pulp. Matsushita and collaborators (1999) found that human pulp cells expressed VEGF spontaneously and

in response to lypopolysacharide (LPS) and pro-inflammatory stimulants *in vitro.* The same authors conducted a study that suggests that VEGF promotes chemotaxis and proliferation of human pulp cells (Matsushita, 2000).

VEGF upregulation will result in enhanced angiogenesis. Thus VEGF might be beneficial in pulp healing processes that require increased blood supply. However, this will only be possible if cells are available for this response. We have previously shown that adhesive resins induce apoptosis of both odontoblasts, and undifferentiated pulp cells. Furthermore, VEGF expression will make existing blood vessels more permeable. The net effect of VEGF expressions is an increase in intra-pulpal pressure and pulp tissue might not capable be capable of surviving the consequences of this pressure. This process may contribute to advancement to pulp necrosis.

Reactive dentinogenesis is mediated by upregulation of odontoblast synthetic and secretory activities from a relative dormant state during physiological dentinogenesis (Linde *et al.,* 1993). Recently, Roberts-Clark and Smith showed that dentine matrix is not an inert dental hard-tissue, but rather a potential tissue store house of a cocktail of bio-active molecules. Previously have been reported that a number of growth factors are sequestered within the dentine matrix (Finkelman *et al.* 1990; Cassidy *et al.* 1997; Pearce *et al.* 1996). Release of growth factors may account for the increased local angiogenesis at sites of dental tissue repair (Baume *et al.,* 1980, Schroder *et al.,* 1985)

We conclude that VEGF is upregulated in some cell populations of the dental pulp, noticeably the odontoblasts in response to an adhesive resin. In a fashion similar to the brain tissue, the dental pulp is restricted within rigid walls. If VEGF plays a role in dental pulp tissue responses, the use of VEGF-blocking antibodies in situations of

uncontrolled vascularization may help to prevent over-exuberant angiogenesis in the confined space of the pulp chamber. It might be expected that anti-angiogenic agents would aid for the decrease blood flow leading to transient normalization of blood vessel permeability and density.

Conclusions

1) Exposure to HEMA resulted in up-regulation of VEGF expression by odontoblast-like cells (MDPC-23) and mouse Macrophages but not in undifferentiated pulp cells (OD-21) or mouse fibroblasts.
2) Exposure to Single Bond resulted in up-regulation of VEGF expression by odontoblast-like cells (MDPC-23) and mouse Macrophages but not undifferentiated pulp cells (OD-21) or mouse fibroblasts.

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CONCLUSION

Adhesive resins have been used to reduce microleakage, postoperative sensitivity and the incidence of recurrent caries. Vital pulp therapy aims the treatment of reversible pulpal injuries by the application of a protective liner or base. Previous studios have questioned the biocompability of bonding agents applied directly to dental pulp cells. The results described in Chapter II are in agreement with Costa *et al* (2000). They demonstrate that single bond induced apoptosis of pulp cells and should not be recommended for vital pulp therapy.

Dental pulp cells, as any other cell in the body, depend on homeostasis of extracellular matrix to remain viable and functional. If this equilibrium is lost, even a modest increase of fluid volume may raise tissue pressure, compress blood vessels, and lead to ischemia and pulp necrosis. If VEGF is upregulated when bonding agents are applied to dental pulp cells, it may increase vascularization of dental pulp tissues, increase vascular permeability and lead to edema and necrosis, because dental pulp has a relative low-compliant environment due to its enclosure between rigid dentin walls, where there is minimum chances of expansion. Therefore the upregulation of VEGF expression might result in additional damage to dental pulp cells.

Our results suggest that an adhesive resin can cause pulp tissue damage through, at least, two mechanisms:

1. Direct cytotoxicity: Adhesive resin causes apoptosis of dental pulp cells; therefore it impairs pulp healing and dentin regulation.
2. Indirect effect: Adhesive rosin upregulates VEGF expression of dental pulp cells. Upregulated VEGF has been correlated with increased tissue

microvessel density and permeability, when is translated into enhanced interstitial pressure. Due to the confinement of the pulp tissue within non-expanding dentin walls, the increase in pressure may result in disruption of pulp homeostasis and additional death of both cells *in vivo*.