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Case Report

Determination of Protective Effect of TGF- β and IGF-1 in Stimulation of Cysteine/Glutamate Exchange in Cultured Dental Pulp Cells

Dr. Pranav Vaid[1*], Dr. Pratiksha A Srivastava (PGT)[2], Dr. Kumar Ravishankar (MDS)[3], Dr. Mamta Singh (MDS)[4], Dr. Meenakshi M. Singh (PGT)[5], Dr. Saurabh Kumar (PGT)[6]

[1]Dental Surgeon Subharti Dental College, Subharti University Subhartipuram, Delhi Meerut Haridwar Bypass, Pin Code-250005
[2]Department of Prosthodontics, Crown and Bridge Subharti Dental College and Hospital, Swami Vivekanand Subharti University, Subhartipuram, Delhi-Meerut-Haridwar Bypass, Uttar Pradesh India
[3]Assistant Professor Department of Periodontology, Aditya Dental College, Beed- 431122 Maharashtra India
[4]Assitant Professor Department of Periodontics, Kothiwal Dental College & Research Centre, Mora Mustaqueem, Kanth Road, Moradabad, 244001, India
[5]Department of Pedodontics and Preventive Dentistry Kothiwal Dental College & Research Centre, Mora Mustaqueem, Kanth Road, Moradabad, 244001, India
[6]Department of Periodontics, Kothiwal Dental College & Research Centre, Mora Mustaqueem, Kanth Road, Moradabad, 244001, India

***Corresponding Author**
Dr. Pranav Vaid

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Abstract: ***Introduction:*** Two important growth factors namely transforming growth factor- β (TGF-β) and Insulin-like growth factor (IGF-1) are known to provide protection to dental pulp cells when cultured against the toxicity induced by the composite materials e.g. Durafill VS and Flow Line. ***Aim:*** Protective mechanism of IGF-1 and TGF-β is mainly via enhanced endogenous antioxidant activity because the toxicity of composite materials is mainly governed by oxidative stress. The present study was planned to investigate the protective effect of growth factors. ***Methods:*** Cultured dental pulp cells were used in current study to determine the mechanism of the protective effects of IGF-1 and TGF-β and their role of cysteine/glutamate exchange (system xc) focusing on the glutathione system. ***Results:*** The toxicity of Durafill VS and Flow Line was debilitated by the addition of glutathione monoethylester, suggestive of a specific role of anti-oxidant glutathione. In support of this hypothesis, it was found that IGF-1 and TGF-β had protective effect against the toxic effect of buthionine sulfoximine (glutathione synthesis inhibitor). Cellular cysteine levels are considered to be the limiting factor in glutathione production therefore, effects of IGF-1 and TGF-β were tested on cysteine uptake which were found to stimulate system xc– guided cysteine uptake. Furthermore, they dissipated the glutathione depletion induced by Durafill VS and Flow Line. ***Conclusions:*** The results were suggestive of protective role of IGF-1 and TGF- β through system xc– guided cysteine uptake stimulation, leading to maintenance of cellular glutathione. This novel role of growth factors IGF-1 and TGF-β helps in preventing toxicity of restorative dental materials and also in the general functioning of dental pulp cells.

Keywords: Cysteine, dental pulp, glutathione, system xc-, toxicity.

INTRODUCTION

Prevention of pulpal cell death after environmental exposure is of utmost importance not just for endodontists but for general dentists as well. Traumatic injuries, rampant caries, or aggressive dental procedures may lead to death of dental pulp cells [1]. Root canal therapy is needed for removal of such damaged pulp. In an attempt to save pulpal tissue when exposed, pulp-capping therapy is also a good treatment option [2]. These procedures mainly intend to stimulate dentin bridge formation over the pulp followed by restoration. Materials containing Calcium hydroxide (Ca[OH]2) are largely used as pulp capping materials due to their ability to stimulate formation of reparative dentin [3]. Reports have provided evidence of Ca (OH)2 being toxic to pulp cells [4]. Success rate of pulp-capping treatments is however variable [5]. Therefore, the effectiveness of such treatment is doubtful [1]. Undoubtedly, better treatment modalities are

required. These days mineral trioxide aggregate (MTA) is in use which was originally developed as a root-end filling material, has gained tremendous popularity as a pulp capping material [6]. However, a long setting time of approximately 4 hours, is a limiting factor impacting its usefulness as a pulp-capping agent [7], and its effects in long-term has yet to be fully analyzed. An adjunct therapy which can be used as potential alternative is using growth factors for pulp- capping. Growth factors are natural proteins that cause alteration in cell proliferation, their differentiation, maturation rate and survival. They are known to stimulate odontoblastic differentiation thus helping in dentin formation [8, 9]. A potential benefit of growth factors over Ca (OH)2 is that they help in reparative dentin formation which is primarily overlying the pulp tissue, whereas Ca(OH)2 effects often has damaging effect on pulp tissue [10]. It has been previously shown that a variety of growth factors can alter differentiation of pulp cell making them resistant to the toxicity induced by restorative materials [11, 12].

The present study intends to determine the mechanism of growth factors (IGF-1and TGF-β) in protection of dental pulp. They were chosen mainly because of their known effects on pulpal cells. TGF-β stimulates formation of primary odontoblasts, helping in dentin formation, is mainly expressed in developing dentition, and enhances activity of alkaline phosphatase and produces mineralization nodules. IGF-1 is known to increase alkaline phosphatase activity in dental pulp cells cultured in vitro and enhances formation of reparative dentin in vivo. Understanding the protective mechanism of each growth factor against toxicity dental material is vital for understanding which growth factors should be used in conjunction with each dental material.

Durafill VS and Flow Line are commonly used composite materials also known to induce oxidative stress resulting in death of pulp cells [12], and this hypothesis served as the basis for exploring the mechanism of protective nature of the growth factors. Highlight of the study is that our focus was mainly on the role of system xc- i.e. cysteine/glutamate exchanger that maintains constant influx of cysteine, which is the rate limiting factor for glutathione production which is an intracellular free radical scavenger. The purpose of the current study is to determine whether growth factors help dental pulp cells to become resistant to cell death or necrosis by up-regulating cysteine/glutamate exchange system.

MATERIALS AND METHODS

Materials

Participants and Human Dental Pulp Cell Cultures

Intact human impacted third molars were procured from adults who signed written informed consent under ethical clearance by the Institutional Review Board. Ten impacted third molars from six patients were used in the current study. Tooth surfaces were scaled and root planed, with a cut around the cementoenamel junction using sterilized diamond stones was made to access the pulp chamber. The dental pulp tissue was removed from the tooth and immersed in a 3 mg/mL collagenase type I solution and 4 mg/mL solution of dispase (protease that cleaves fibronectin, Type IV collagen) for 60 minutes at 37○C [11]. The cells were placed onto 24-well plates that was pre-coated with laminin and poly-D-lysine in Eagle medium supplemented with 20% fetal calf serum/100 mmol/L, L-glutamine/100 U/mL, L-ascorbic acid 2-phosphate/2 mmol/L, penicillin/100 mg/mL and streptomycin. Later it was incubated with 5% CO2 at 37○C. 100 ng/mL of growth factors were then added. Experiments were performed in vitro on 7 to 9 days of cultures.

Preparation of Dental Materials

Flow Line and Durafill VS were specifically prepared in accordance with manufacturer's instruction manual. They were then dispensed on a sterile glass slab for a brief period followed by curing with a visible light curing gun for 60 seconds and cut into pieces of uniform sized. Curing gun used was a product of 3M Unitek.

Exposure of Dental Materials to Cell Cultures

Freshly prepared dental materials were placed into 96-well plates in 250 mL of media similar to plating media except lacking serum. After 24 hours, the media was removed from the 96-well plates and placed on the cells growing on 24-well plates for 6 hours (glutathione assays) or for 48 hours (toxicity assays). For experiments in which glutathione monoethylester was tested, it was present during the 48-hour exposure of the cells to the dental material treated media. The weights of the materials used were the following: Flow Line, 9.5 T .4 mg, and Durafill VS, 10.0 T .4 mg.

Cell Viability Assessment

Cell injury was quantified by the measurement of the reduction of yellow 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product. MTT was added to each well 48 hours after the beginning of insult to the cells. After a 30-minute incubation, the media was removed, and cells were dissolved in dimethyl sulfoxide. The

formation of formazan was measured as the amount of reaction product by absorbance change at a wavelength of 590 nm by using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Levels of formazan formation from cultures exposed to 10 mmol/L of the calcium ionophore A23187 (100% cell death) were subtracted from insult formazan levels, and results were normalized to control [12]. The possibility exists that cultures prepared from different teeth have different properties. To mitigate this potential complication, all experiments include control conditions on the same 24-well plate. Therefore, cell death for each experimental condition is compared with a control from the same pulp source.

14C-Cysteine Uptake

The uptake of cysteine was measured by exposure of cultures to 14C- cysteine (0.1 mCi/mL) for 20 minutes. After the exposure to 14C-cysteine, the cultures were washed three times and dissolved in 1% sodium do- decyl sulfate (250 mL). An aliquot (200 mL) was removed and added to scintillation fluid for counting. Values were normalized to control 14C-cysteine uptake (20-minute exposure to 14C-cysteine without growth factor or dental material treatment).

Monochlorobimane Assay of Cellular Glutathione

Cellular glutathione levels were measured by monochlorobimane (MCB) fluorescence. MCB forms a fluorescent compound when it reacts with glutathione through a reaction catalyzed by glutathione-S- transferase. MCB was added to the media after a 6-hour treatment with dental materials, a time point before gross cell death occurs. After 30 minutes, the cultures were excited at a wavelength of 355 nm and emission measured at a wavelength of 460 nm using a Thermo Labsystems Fluoroskan microplate reader. Background (no MCB added) was subtracted and values normalized to control (MCB but no dental material present).

STATISTICAL ANALYSIS

Statistical calculations of the continuous variables assessed in the studies were performed using one-way analysis of variance followed by the Bonferroni correction post hoc test. Statistics were calculated using SSPS version 18.0. P values <.05 were considered to indicate significant differences.

RESULTS

Durafill VS and Flow Line both caused significant toxicity after 48 hours of exposure as measured by the MTT metabolism assay (Fig. 1). The toxicity of the dental materials was no longer observed in cultures that were treated with IGF-1 or TGF-β (Fig. 1). Because the growth factors can alter the levels of MTT metabolism caused by changes in cell growth and differentiation, the effects of the dental materials are compared with the starting level of MTT metabolism with each growth factor treatment.

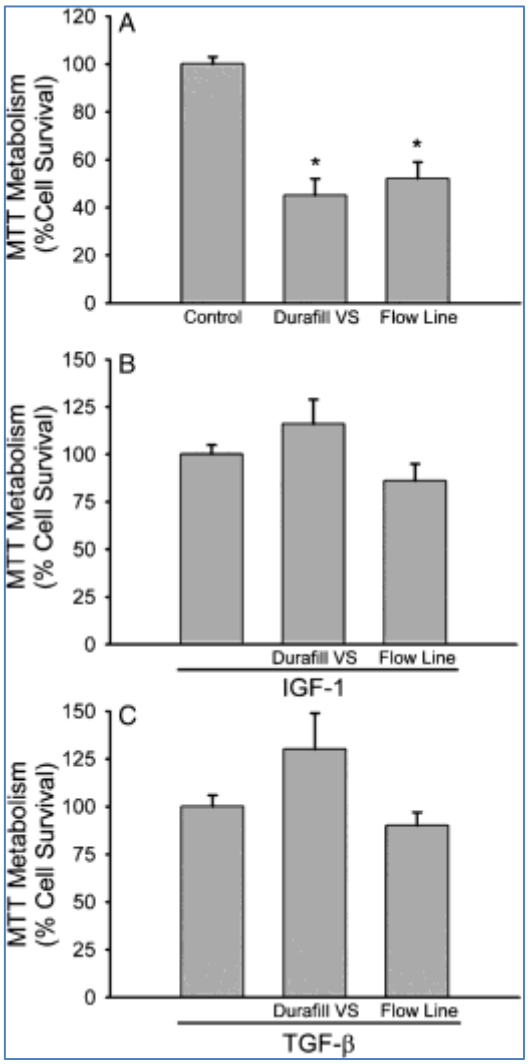

Fig-1: Durafill VS and Flow Line cause significant toxicity to (A) dental pulp cells, which is eliminated by treatment with (B) IGF-1 or (C) TGF-β . Dental materials were incubated in media for 24 hours at which time the conditioned media was placed on the cultures for 48 hours. Bars show % cell survival (mean standard error of the mean, n = 12–16) quantified by the inhibition of MTT reduction. Control represents MTT levels in untreated cultures (no dental materials) and is defined as 100% cell survival. *Significant difference from control (P < .05).

The toxicity of Durafill VS and Flow Line was attenuated by the addition of the cell permeable form of glutathione, glutathione monoethylester (Fig. 2). This finding suggests a specific role for glutathione depletion in Durafill VS and Flow Line toxicity and raises the possibility that the protective effects of IGF-1 and TGF-β may be mediated by enhancing cellular glutathione levels. In support of this idea, we found that IGF-1 and TGF-β were also protective against toxicity induced by buthionine sulfoximine, an inhibitor of glutathione synthesis

(Fig. 2). A potential mechanism for this protection is provided by the result that IGF-1 and TGF-β both increased 14C-cysteine uptake (Fig. 3). The increased cysteine uptake appears to be mediated by system xc- because the selective inhibitor of that system, sulfasalazine, blocked the stimulated uptake (Fig. 3). Because the levels of cellular cysteine are the rate-limiting step for the synthesis of glutathione, it seemed possible that the protective effects of IGF-1 and TGF-β could be caused by an increased cysteine uptake leading to the maintenance of cellular glutathione levels during an insult. Six-hour exposure to Durafill VS and Flow Line caused a significant decrease in cellular glutathione as measured by the fluorescent dye MCB (Fig. 4). At this time point, there was no overt cell death as determined by a lack of release of the cytosolic enzyme lactate dehydrogenase (data not shown), a well-established method of assessing gross cell death. In cultures treated with IGF-1 or TGF-β, Durafill VS and Flow Line still caused a significant decrease in cellular glutathione, but the decrease was attenuated by the growth factor treatment (Fig. 4).

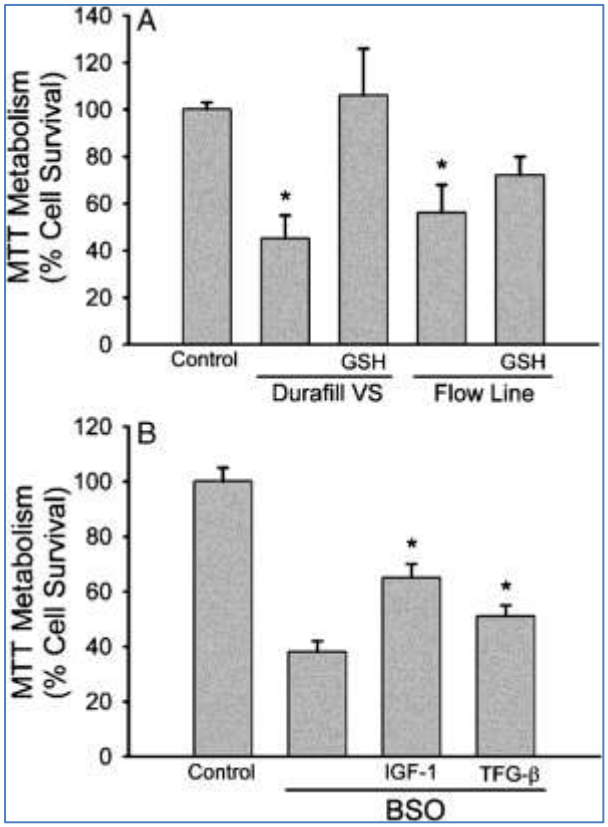

Fig-2: (*A*) The toxicity of Durafill VS and Flow Line is attenuated by the cell permeable glutathione monoethylester (GSH, 100 mmol/L), whereas (*B*) IGF-1 and TGF-b protect against the toxicity induced by the inhibition of glutathione synthesis induced by 1 mmol/L buthionine sulfoximine buthionine sulfoxi- mine. Control represents MTT levels in untreated cultures (no dental materials) and is defined as 100% cell survival. Bars show % cell survival (mean SEM, n = 8–16) quantified by the inhibition of MTT reduction. *Significant difference from control (*P* < .05).

DISCUSSION

The present study shows a novel mechanism by which growth factors stimulate system xc–mediated cysteine uptake in pulpal cells providing resistance against oxidative stress mediated cell death. The main finding of the present study is that protective effect of the growth factors is due to stimulation of system xc- function. This result has broad implications because of the critical effects of system xc- on cell function and survival.

System xc- regulates two important actions: the release of glutamate and the uptake of cysteine and. Highlight of the study is mainly on the role of system xc- i.e. cysteine/glutamate exchanger that maintains constant influx of cysteine, which is the rate limiting factor for glutathione production which is an intracellular free radical scavenger. Multiple processes have implied on the depletion of cellular glutathione such as neurodegenerative diseases like schizophrenia. A vital aspect of the present study is that growth factors are protective for dental pulp cells against insults related to oxidative stress. Oxidative stress is a common terminal step in pathway of toxicity induced by many compounds, including restorative dental materials. Oxidative stress has also been shown to be involved in the death of pulp cells induced by infection. Interestingly, it was found that IGF-1 and TGF-β provide protection against amalgam toxicity as well [11].

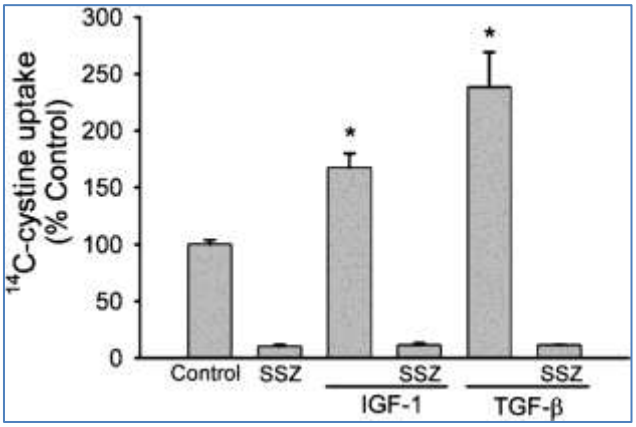

Fig-3: IGF-1 and TGF-β stimulate cysteine-glutamate exchange (system xc-) mediated 14C-cysteine uptake. Sulfasalazine (SSZ, 200 mmol/L) selectively inhibits cysteine-glutamate exchange mediated cysteine uptake. Bars show % 14C-cysteine uptake normalized to control uptake (mean standard error of the mean, n = 12–24). *Significant difference from control 14C-cysteine uptake (P < .05).

Although status of amalgam toxicity mediated by oxidative stress is unclear, mercury toxicity definitely involves oxidative stress. Also, we have found that amalgam toxicity can be induced by the release of zinc, and zinc toxicity involves

oxidative stress. Many dental materials contain zinc, usually in the form of zinc oxide, which may account for their toxicity. Thus, the ability to dissipate oxidative stress–induced cell death is of general importance. The mechanism of protection by IGF-1 and TGF-β appears to be through an increased uptake of cysteine leading to better maintenance of cellular glutathione during oxidative insults. Although there was still a decrease in glutathione levels caused by Flow line and Durafill VS after growth factor treatment, the decrease was attenuated. In three of the conditions (Flow Line after IGF-1 or TGF-β treatment and Durafill VS after IGF-1 treatment,) the decrease in glutathione was significantly attenuated ($P < .05$), whereas in one of the conditions (Flow Line after TGF-β treatment) there was only a trend toward less glutathione decrease with growth factor treatment. Growth factors have the potential for multiple uses involving dental pulp cells. They have the ability to differentiate pulp cells into odontoblast-like cells and may potentially play a role in total tooth regeneration. They may also be useful for the specific application of stimulating the formation of reparative dentin, making them potentially useful in pulp capping procedures.

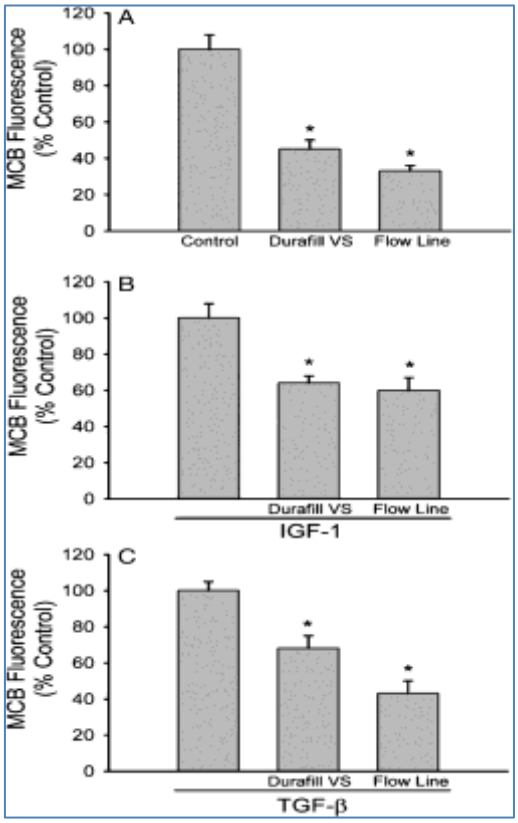

Fig-4: The decrease in cellular glutathione levels induced by (A) Durafill VS and Flow Line is attenuated by treatment with (B) IGF-1 or (C) TGF-β. The total reduced glutathione is determined by MCB fluorescence. Bars show % MCB fluorescence normalized to control fluorescence (no dental material) (mean T SEM, n = 8–16). *Significant difference from control ($P < .05$).

CONCLUSION

Both IGF-1 and TGF-β have shown to stimulate formation of reparative dentin in experimental animal models. Also, increased in TGF-β expression at the odontoblastic-subodontoblastic zone in teeth having irreversible pulpitis is suggestive of involvement of endogenous TGF-β in dentinal repair during pulpitis. The finding that IGF-1 and TGF- β upregulates cysteine uptake, while also mitigating oxidative stress– induced apoptosis and depletion of glutathione is suggestive that they may have broad protective effects. Because oxidative stress is a common final mechanism of cell death under multiple conditions, the results suggest that IGF-1 and TGF-β should be considered in situations where growth factors can be used to differentiate pulpal cells because those cells would be undoubtedly more resistant to cell death in varied conditions. Ideally pulp-capping treatment should be a combination of growth factors and a bioinert compound.

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