Caries Induced FGF-2 Expression in Human Dental Pulp

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DEDICATION

To Luis y Yolanda, for being exemplary parents and giving me unconditional support through out life. Nothing I have done would have been possible without you two.

To my grandfather Elias. For being my companion till the end. I will continue to dedicate efforts and accomplishments to you. Thanks for being around when I need someone to talk to. Still remember the old days. R.I.P.

To all patients. May science one way or the other continue to help the ones in need.

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ABBREVIATIONS

Commonly Used Abbreviations

FGF-2	Basic Fibroblast Growth Factor
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet Derived Growth Factor
PIGF	Placenta Derived Growth Factor
TGF	Transforming Growth Factor
TNF-α	Tumor Necrosis Factor-Alpha
TNF-β	Tumor Necrosis Factor-Beta
GF	Growth Factor
PDL	Periodontal Ligament
CEJ	Cemento-enamel junction
PCR	Polymerase Chain Reaction
RT	Reverse Transcriptase
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-Linked Immunosorbent Assay
Ab	Antibody

EC	Endothelial Cell
SD	Shallow Decay
DD	Deep Decay
C	Control
PBS	Phosphate Buffered Solution
ETOH	Ethanol
Hrs	Hours
Min	Minutes
Sec	Seconds
Ref.	Reference No.
#	Number
IRB	Institutional Review Board
NA	Neutralizing Antibody
HDPF	Human Dental Pulp Fibroblasts
MMPs	Metalloproteinases

TABLE LEGENDS

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ABSTRACT

Fibroblast growth factor (FGF-2) plays a significant role in wound healing and increases local angiogenesis. Increased blood vessel permeability and angiogenesis are commonly found in caries-induced pulpitis. To date, there are no studies evaluating the presence of FGF-2 in pulp tissues from carious teeth. The purpose of this study is to evaluate the presence of FGF-2 in the pulp of carious teeth as compared to non-carious teeth. Thirty healthy patients (18-55 y/o) scheduled for multiple extractions were selected. One sound tooth and one carious vital tooth were collected from each patient. Teeth were grouped as deep caries (15) and shallow caries (15). Teeth were split, the pulp tissue removed and cut in half longitudinally. Protein and RNA were extracted and FGF-2 expression measured by ELISA, Reverse transcriptase polymerase chain reaction (RT-PCR) and Real-Time PCR. Either One-way analysis of variance (ANOVA) or the student *t*-test were used for statistical analysis. The analysis of protein expression evaluated by ELISA showed no statistical significant difference when comparing FGF-2 expression in all shallow or deep decayed samples to non-decayed samples. Densitometric analysis of RT-PCR showed similar trends of increase or

decrease of FGF-2 expression as analyzed by ELISA in 10 of 15 (67%) in the shallow decay group and 8 of 15 (54%) in the deep decay group. Quantitative analysis measured by Real Time PCR did not show a statistically significant difference when comparing FGF-2 expression in the non-carious versus the carious group. Age, sex and tooth type did not appear to be determinant factors in the up-regulation or down-regulation of FGF-2 within the shallow decay or deep decay group. The results of this study revealed that human pulp tissue contains fibroblast growth factors (FGF-2) both in health and during inflammation. These findings provide an insight for understanding the role of FGF-2 in the mechanism of caries progression and inflammation. The results of this study revealed that human pulp tissue contains fibroblast growth factor (FGF-2) both in health and during inflammation. These findings provide an insight for understanding the role of FGF-2 in the mechanism of caries progression and inflammation.

SECTION I

1. Background and Significance

Growth factors are diffusible cell-signaling molecules that pass between the epithelial and mesenchymal compartments of the tooth germ and are responsible for interactions during tooth development. These interactions determine the early morphogenic events in tooth development (Cobourne *et al.,* 2003) as well as the later processes that give rise to odontoblasts (Ruch *et al.,* 1995) and ameloblast differentiation (Unda F *et al.,* 2001).

The importance of growth factors in mediating the cellular response to injury in the dentin-pulp complex is well recognized. Several growth factors are reportedly sequestered in the dentin matrix from where they may be released during the repair process (Smith, 2002). The process of reactionary and reparative dentinogenesis at sites of dental injury are responsible for secretion of tertiary dentin matrices, which either increase the dentin barrier between the site of injury and the underlying cells in the unexposed pulp or provide a dentin bridge across the exposed pulp (Smith *et al.,* 2002). Growth factors may be key molecules in the signaling of the biological events responsible for these repair processes (Smith *et al.,* 2003).

Vascular endothelial growth factor (VEGF), platelet derived growth factor (PDFG) and Fibroblast growth factor (FGF-2), are among the angiogenic growth factors identified in human dentin matrix and pulp tissue (Clark *et*

al., 2000; Hung *et al.,* 2007). Bacterial toxins such as lipoteichoic acid and lipopolysacharide increased the expression of angiogenic growth factors (VEGF) in odontoblast like cells (Telles *et al.,* 20003; Botero *et al.,* 2006). Hung *et al.,* 2007 demonstrated how human pulp fibroblasts secrete VEGF, PDGF and **FGF-2** after mechanical injury (Hung *et al.,* 2007).

The vitality of the dentin-pulp complex, both during tissue homeostasis and after injury is dependant on pulp cell activity and the signaling processes which regulate the behavior of these cells. Growth factors are peptide molecules that transmit signals between cells functioning as stimulators and/or inhibitors of growth as well as modulators of differentiation amongst other roles (Smith *et al.,* 2003). The distribution of these growth factors between the organic and inorganic tissue compartments of the dentin matrix may reflect the manner in how they become sequestered within the tissue and will also determine their potential release characteristics (Clark *et al.,* 2000)**.** The higher concentrations of most of these growth factors in the soluble compartments of dentin will allow ready release during dental caries (Clark *et al.,* 2000).

Diffusible angiogenic growth factors including (VEGF, **FGF-2**, PDGF, TGF-β) are regulated in the pulps of orthodontically moved teeth as compared with controls, where they might mediate local angiogenic

responses to tissue events (Derringer *et al.,* 1998; Derringer *et al.,* 1996). Derringer *et al,* 2004 showed by means of human dental pulp and rat aorta co-culture assay how these growth factors not only play a role in angiogenesis but at the same time function effectively when acting in combination (Derringer *et al.,* 2004). Release of FGF-2 could account for the increased local angiogenesis observed at sites of dental tissue repair after dental caries (Baume *et al.,* 1980; Schroder *et al.,* 1985; Smith *et al.,* 1990) or traumatic injury (Baume *et al.,* 1980; Schroder *et al.,* 1985; Smith *et al.,* 1990).

The vascular responses that are observed in teeth with deep caries and the edema mediated by the potent angiogenic and vascular permeability factor VEGF may be detrimental to the dental pulp health (Botero *et al.,* 2006). Thus, the overall response in the pulp is likely to be a summation of the effects arising from release of growth factors from the dentin matrix (Sloan and Smith, 1999) and more local secretion from the cells in the pulp itself (Sloan *et al.,* 2000).

The rationale for this investigation emerged from a review of the literature pertaining to the presence of fibroblast growth factor (**FGF-2**) during pulpitis. Currently there is no information of FGF-2 and its correlation with the progression of carious lesions into the pulp. It would be of value to

assess the presence of FGF-2 in the pulp. Additionally, the expression FGF-2 should be evaluated in sound teeth and teeth with dental caries. The results of this study could provide relevant information to understand the regeneration and possible future therapeutic approaches.

2. PURPOSE AND HYPOTHESIS

PURPOSE

To demonstrate and measure the presence of FGF-2 in carious dental pulp as compared to non-carious dental pulp from teeth collected from the same patient.

Hypothesis:

The pulps of teeth with carious lesions show higher levels of FGF-2 expression when compared to the pulps of non-carious teeth.

Null Hypothesis:

The pulps of teeth with carious lesions do not show higher levels of FGF-2 expression when compared to the pulps of non-carious teeth.

3. LITERATURE REVIEW

Growth Factors

Growth factors are naturally occurring biological mediators that act as signals between cells. They modulate cell growth and differentiation (Smith *et al.,* 2003). As such they play a central role in controlling cell behavior and activity. Growth factors may demonstrate a degree of specificity in terms of the cells they act upon, although some are more versatile and act on numerous cell types. The dose dependency of their effects also varies, however, one of the characteristic features of these molecules is their potency at very low concentrations, typically in the picogram range (Smith *et al.,* 2003). While many are named after the original source they were isolated from this may be misleading because a more widespread distribution has often been since demonstrated (Smith *et al.,* 2003).

Growth factors act through their interaction with specific receptors on the cell surface. Binding to these receptors leads to a chain of intracellular signals, the result of which is transmission of the signal to the cell nucleus (Matsuda *et al.,* 1992; Graves and Cochran, 1994). It is through their effects on gene expression in the cell nucleus, mediated by transcription and other factors, that growth factors influence cell behavior and activity. This transcriptional control of gene expression can have far-reaching effects both

in terms of intra- and extra-cellular events. Thus, growth factors may regulate genes controlling cell proliferation, cell differentiation, or the secretory products of the cell. (Smith *et al.,* 2003).

Growth Factors in Tooth Development

Growth factors are responsible for signaling many of the key events in tooth morphogenesis and differentiation (Cobourne *et al.,* 2003) and recapitulation of these processes after dental injury allows tissue regeneration (Smith *et al.,* 2002).

During the late bell stage of tooth development, the inner dental epithelium and its associated basement membrane signal the peripheral cells of the dental papilla to differentiate into odontoblasts (Ruch *et al.,* 1995). Growth factors, particularly of the transforming growth factor-β (TGF-β) family, appear to be important molecules mediating this signaling of odontoblast differentiation (Bègue *et al.,* 1992). Secretion of these growth factors by the inner dental epithelium and their sequestration within the dental basement membrane for presentation to the dental papilla cells for signaling of odontoblast differentiation, provide the temporo-spatial control of these processes in the tooth germ (Ruch *et al.,* 1995). However, in the mature tooth, similar processes must occur to allow differentiation of a new

generation of odontoblast-like cells for dentin bridge formation (Bègue *et al.,* 1992).

Growth Factors in the Dentin-Pulp Complex

Dentin chips arising from operative debris have been known to be auto-inductive for reparative dentinogenesis (Seltzer *et al.,* 1990). Experimentally, demineralized dentin matrix and isolated dentin matrix components are also capable of inducing reparative dentinogenesis and bridge formation at sites of pulp exposure (Anneroth *et al.,* 1972; Nakashima *et al.,* 1989; Nakashima *et al.,* 1990; Tziafas *et al.,* 1990; Smith *et al.,* 1990) as well as ectopic bone formation (Butler *et al.,* 1977). This implies that dentin matrix contains bioactive components and is not as inert as sometimes presumed. The collagenous and non-collagenous proteins of dentin have been well reviewed (Linde *et al.,* 1993; Butler *et al.,* 1998; Butler *et al.,* 2002), but the emphasis has been on the quantitatively more important components. It has now been possible to identify a number of growth factors in dentin matrix, which, while quantitatively minor components, may have potential biologic effects. The origin of these growth factors in dentin matrix is probably largely the odontoblast cell (Sloan *et al.,* 2000). Once incorporated within dentin matrix, these growth factors become " fossilized " and retain their biological activity through the protection

offered by their interaction with dentin extracellular matrix components (Sloan *et al.,* 2000).

Growth factors identified within human dentin matrix include VEGF, FGF-2, TGF-β, PDGF, epidermal growth factor (EGF), insulin growth factor -1 (IGF-1), insulin growth factor-2 (IGF-2) and placenta growth factor (PIGF) (Clark and Smith, 2000; Finkelman *et al.,* 1990). Dentin matrix therefore contains a cocktail of bioactive molecules with potent cell signaling properties, which may be released into the pulp environment during tissue injury (Clark and Smith, 2000). While the sequestration of growth factors within dentin matrix (Clark and Smith, 2000) provides one possible pool of these cell-signaling molecules in the injury situation, fibroblasts and other pulp cells may be other sources (Hung *et al.,* 2007; Sloan *et al.,* 2000). Both the physiological cell populations of the pulp and the inflammatory cells, which locally infiltrate the tissue at sites of injury, also express a number of growth factors and may contribute to the overall tissue response (Sloan *et al.,* 2000). VEGF, FGF-2, PDGF, TGF-β, and hepatocyte growth factor (HGF) are among the growth factors isolated in human pulp tissue (Derringer *et al.,* 2004; Hung *et al.,* 2007; Dale *et al.,* 2002). Although these sources of growth factors may not become " fossilized " in the same way as those deriving from the dentin matrix because of the greater turnover

in the extracellular matrix of the pulp soft tissue, they will nevertheless contribute to the more immediate cellular responses (Sloan *et al.,* 2000).

Vascular Endothelial Growth Factor (VEGF)

VEGF is a key regulatory factor in the control of vascular permeability and angiogenesis (Ferrara *et al.,* 2002). The ability of VEGF to enhance vascular permeability is estimated to be 50,000 times higher than histamine (Ferrara *et al.,* 2003). Vascular endothelial growth factor is originally a basic 45-kDa heparin-binding glycoprotein. VEGF proteins may become available for the endothelial cells (ECs) by at least two different mechanisms: either by alternative splicing (originating diffused proteins) or by protease activation and longer isoform cleavage (Ferrara *et al.,* 2002). The VEGF family includes six known members: VEGF-A, B, C, D, E and Platelet derived growth factor (PDGF), along with two growth factor receptors (VEGFR-1 and VEGFR-2) expressed on the vascular endothelial cell surfaces (Ferrara *et al.,* 2003). It has been hypothesized that an increase of VEGF expression by the odontoblasts during the bacterial challenge in deep caries might contribute to vascular changes in the pulp (Botero *et al.,* 2006).

Fibroblast Growth factor (FGF)

FGF is a heparin-binding protein. Fibroblast growth factor was found in pituitary extracts by Armelin in 1973 (Armelin *et al.,* 1973) and then was

also found in a cow brain extract by Gospodarowicz in 1974 and tested in a bioassay which caused fibroblasts to proliferate (first published report in 1974) (Gospadarowicz *et al.,*1974). The FGF family consists of nine structurally related polypeptides: two prototypes, acidic FGF (FGF-1) (Jaye *et al.,* 1986) and basic FGF-2 (FGF-2) (Abraham *et al.,* 1986 a), and seven additional members, FGF-3 (Dickson *et al.,* 1984), FGF-4 (Sakamoto *et al.,* 1986), FGF-5 (Zhan *et al.,* 1988), FGF-6 (Marics *et al.,* 1989), FGF-7 (Rubin *et al.,* 1989), FGF-8 (Tanaka *et al.,* 1992), and FGF-9 (Miyamoto *et al.,* 1993). FGF-2 functions by receptor binding (Ornitz, 2000). The family of FGF receptors includes four members: FGFR1, FGFR2, FGFR3, and FGFR4 (Lee *et al.,* 1989; Dionne *et al.,* 1990; Keegan *et al.,* 1991; Partanen *et al.,* 1991).

FGFs are considered multifunctional proteins with a wide variety of effects. They are most commonly mitogens but also have regulatory, morphological, and endocrine effects. They have been alternately referred to as "pluripotent" growth factors and as "promiscuous" growth factors due to their multiple actions on multiple cell types (Arese *et al.,* 1999; Vlodavsky *et al.,* 1990). Promiscuous refers to the biochemical and pharmacological concept of how a variety of molecules can bind to and elicit a response from single receptor. In the case of FGF, four receptor subtypes can be activated by more than

twenty different FGF ligands. Thus, the functions of FGFs in developmental processes include mesoderm induction, antero-posterior patterning, limb development, neural induction and neural development (Green *et al.,* 1996) and in mature tissues/systems angiogenesis, keratinocyte organization, and wound healing processes.

FGF-1 and FGF-2 are among the fibroblast growth factor most extensively studied and have been shown to induce angiogenesis (Baffour *et al.,* 1992). During cell injury and repair FGF-2 is involved in repair and tissue regeneration (Detillieux *et al.,* 2004). As this process requires angiogenesis, FGF-2 has a vital regenerative function in these events. Vessels generated by VEGF tend to be "capillary like and leaky" but those produced by FGF-2 appear to be more mature (Abo-Auda and Benza, 2003). FGF-2 affects smooth muscle cells, fibroblasts, and ECs (Slavin, 1995). The ECs are stimulated to produce plasminogen activators and matrix metalloproteinases causing extracellular breakdown and vascular remodeling. In animal studies, increased EC proliferation, increased collateral vessel density, higher perfusion pressure and improved regional blood flow have been noted, showing the ability of FGF-2 to induce angiogenesis (Baffour *et al.,* 1992).

FGF-2 in the Dental Pulp

The literature pertaining to this specific angiogenic growth factor (**FGF-2**) has shown its presence in pulp tissue and its potentiating and synergistic effects on other major angiogenic inducers (VEGF and TGF-β) (Derringer *et al.,* 2004). There are, however, no studies evaluating their presence during inflammation in pulp tissue. Inflamed pulp tissues enhance the expression of inflammatory mediators. Chu *et al.,* 2004 reported that pro-inflammatory cytokines can induce VEGF mRNA gene expression in human pulp, which may partially contribute to destruction of pulpal tissues through the expansion of the vascular network coincident to progression of inflammation.

Further studies are needed to characterize the biological actions of FGF-2 in human inflamed pulp tissue. Elucidation of the biological roles of FGF-2 may provide information useful in the generation of an agent for the treatment of injury of the dentin/pulp complex. This could be utilized for future research in order to modulate growth factor expression, design therapeutic blockages and/or induce revascularization of dental pulp tissue when needed.

SECTION II

1. Introduction

The formation of new blood vessels is a complex, multistage process closely regulated by molecules that induce or inhibit the development of new blood vessels. The formation of new blood vessels from pre-existing microvasculature (angiogenesis), occurs during tissue repair (Hertig, 1935) embryonic development, chronic inflammation and tumor growth. It is a complex process, which includes: extracellular matrix remodeling, release of proteolytic enzymes, endothelial cell migration, proliferation, capillary differentiation and the formation of anastomosis (Folkman *et al.,* 1992). This process is regulated by the interplay of numerous cytokines and growth factors (Booth *et al.,* 1998).

The intensity and duration of tooth injury following dental caries or trauma determines the nature of the pulpal response. Under pathological conditions, angiogenesis is a key step in the healing sequence of the dental pulp with hard tissue formation. Injured pulp cells secrete angiogenic growth factors to stimulate angiogenesis, which precede the reparative dentin formation (Hung *et al.,* 2008). Among the factors released after injury, FGF-2 is known to stimulate angiogenesis in vivo (Gerwins *et al.,* 2000) and may act as a mitogen for pulp progenitor cells (Nugent *et al.,* 2000). It is well established that injured endothelial cells release signaling molecules (FGFs) which are

involved in the initiation of the inflammatory reaction and the healing process (Martin, 1997) and seem to be involved in the recruitment of odontoblast-like cells at the injury site (Mathieu *et al.,* 2005). FGF-2 plays a role in the initial inflammation during wound repair in vivo earlier that in the absence of FGF-2 (Murakami *et al.,* 1999), and also participates in the subsequent tissue healing. Furthermore, an in vitro study demonstrated that FGF-2 up-regulated (increased) early neutrophil adhesive interaction with fibroblasts and later down-regulated (decreased) it (Zhang *et al.,* 2001).

The secretion of FGF-2 in healthy and mechanically injured pulps has been shown (Derringer *et al.,* 2004; Hung *et al.,* 2007). However, the expression of FGF-2 in human inflamed pulp tissue has never been demonstrated. Here we present the results of the assessment and quantification of FGF-2 expression in carious dental pulp as compared to non-carious dental pulp. We believe that these results could provide an insight for understanding the role of FGF-2 in the mechanisms of caries progression and inflammation.

2. MATERIALS AND METHODS

A total of 60 teeth (30 carious and 30 non-carious) were obtained from 30 healthy patients (18-55 years old) scheduled for multiple extractions in the Department of Oral Surgery at the University of Michigan, School of Dentistry, Ann Arbor, with signed patient's consent and University's institutional review board (IRB) approval. Both decayed and non-decayed teeth were obtained from the same patient source (matched pairs).

Any history of pain was recorded and vitality evaluated by cold test if pulpal diagnosis needed to be confirmed (Hygienic, Inc., Akron, OH). Teeth with necrotic pulps and/or periapical pathology or an open apex were not included. After extraction, the teeth were immediately placed into sterile Transport Medium i.e. high glucose DMEM (Sigma Chemical Co.) supplemented with L-glutamine (Gibco), penicillin, streptomycin (Gibco) and Amphotericin B (Sigma), on ice.

After total sample collection, teeth within the carious category were separated and grouped as shallow caries (15) (Fig.1) or deep caries (15) (Fig. 2) based on the depth of decay and its proximity to the pulp chamber.

Assignment to these groups was made during the vertical sectioning of the teeth. Teeth with decay in contact with pulp tissue or within 2 mm of the pulp chamber were

categorized as deep decay. Teeth with more than 2 mm of sound dentin separating the leading edge of the carious lesion from pulp tissue were categorized as shallow decay.

Figure 1. A sectioned tooth with the 'Shallow Decay' showing sound dentin separating the carious lesion from the pulp chamber.

Figure 2. A tooth sectioned with 'Deep Decay' showing the carious lesion in contact with the pulp.

PREPARATION OF SPECIMENS

The tooth surface was disinfected using a sterile gauze swab soaked in 70% ethanol. Tissue and deposits adhering to the outside of the tooth was removed with a sterile curette (Miltex 7-540 / G11-12).

Each tooth was affixed to a wooden block (3 cm x 3 cm x 1.5 cm) with Super Glue (Elmers Products, Columbus, OH) (Fig. 3). These blocks were then mounted on an Isomet Low Speed circular saw (Model 650, South Bay Technology, Inc., San Clemente, CA) (Fig. 3). The teeth were sectioned

vertically (Fig. 4) with a lapidary blade 303 Series (MK-303 Professional, MK Diamond Products Inc., Calais, ME) while spraying sterile phosphate buffered saline (PBS-1X; Gibco) as a coolant. The saw was sterilized and washed with 70% ethanol and sterile PBS before sectioning of the teeth.

After each tooth was sectioned the pulp was removed intact using sterile blunt instruments. The harvested pulp was then vertically cut into halves. Each half was placed in a labeled eppendorf tube. Half of the tubes contained lysis buffer for protein (NP-40 Reagent/ eBioscience). The rest of the tubes contained RNA lysis buffer (Trizol Reagent/ Invitrogen). All specimens were then stored at -80° C.

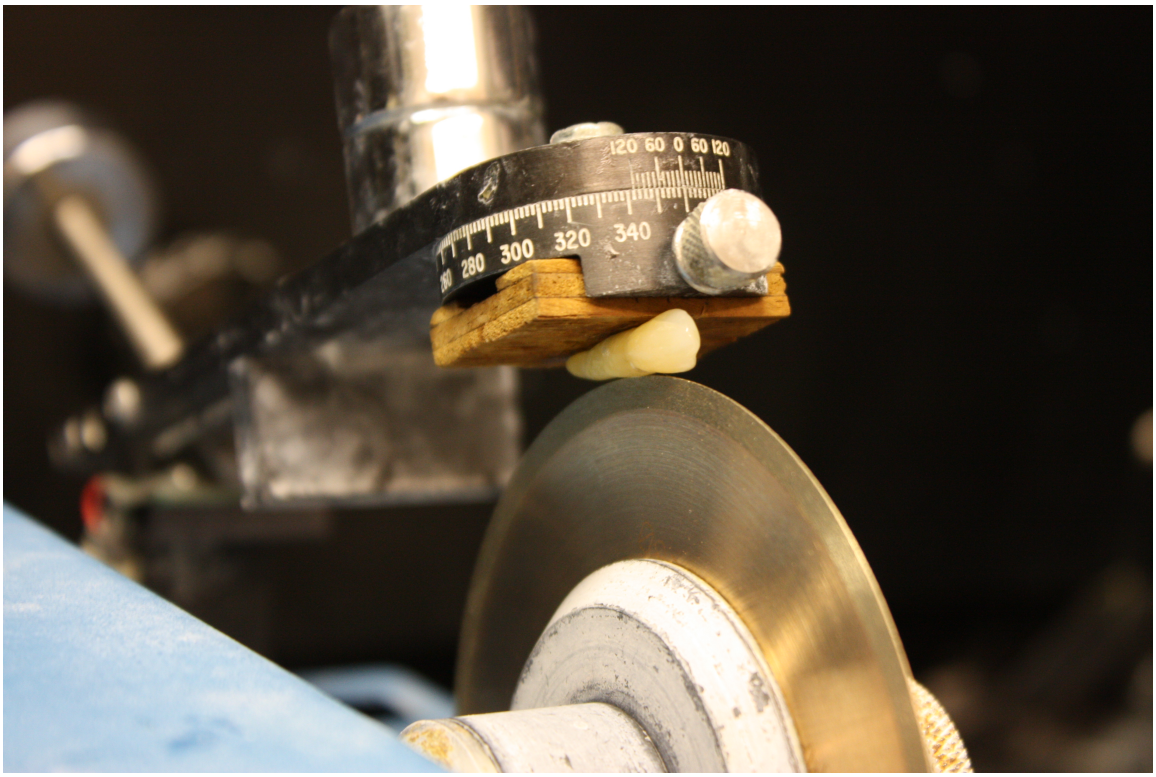


Figure 3. Diamond Wheel Saw Model 650 (Southbay Technologies, San Clemente, California) with a tooth glued to a wooden mounting in the cutting jig. Sectioning was carried out in a fume chemical hood under aseptic conditions.

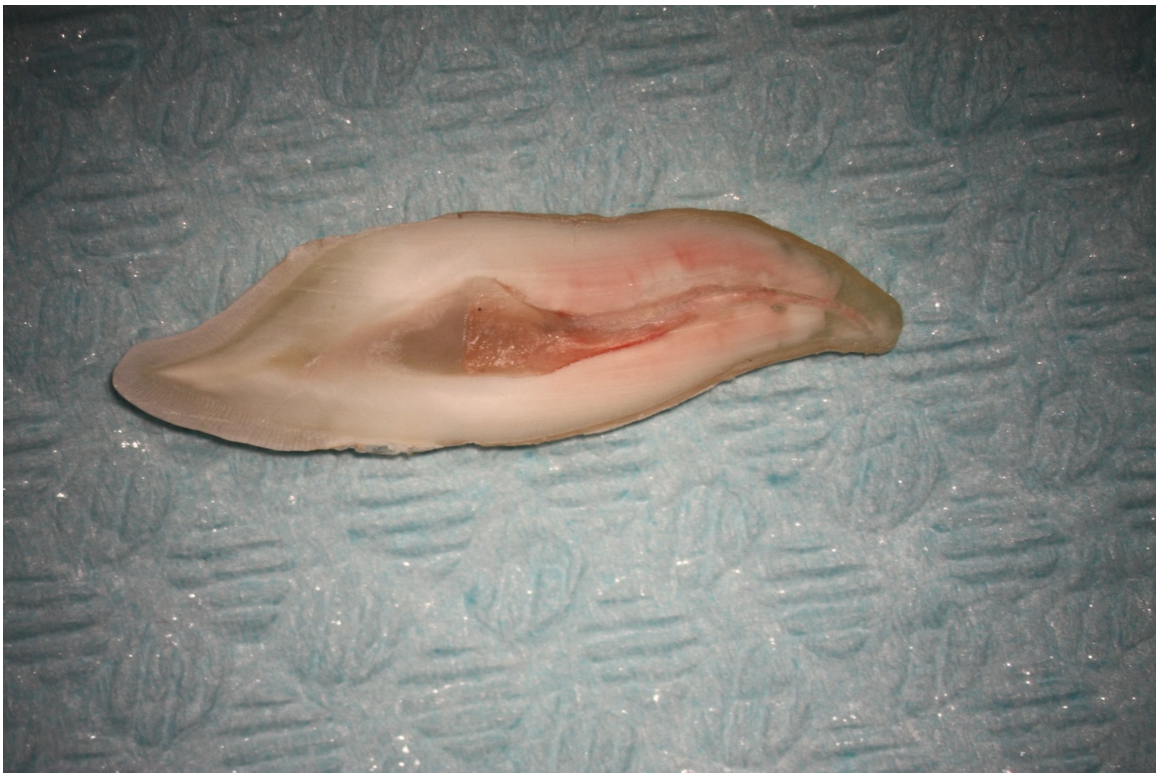


Figure 4. A caries free tooth longitudinally prior to removing the pulp.

ELISA

The preparation of protein lysate from the first set specimens stored in buffered lysate solution was performed following standard protocols (Appendix, Section B). Prior to FGF-2 protein analysis, total protein concentration from each sample was standardized to 200 μg/ml. Enzyme linked immunosorbent assay (ELISA) (Quantikine Human Kit; R & D Systems, Minneapolis, MN, USA) was used to measure the concentration of **FGF-2** in the specimens stored in. A standardized solution of FGF-2 was made with 2 ml of calibrator diluent RD5-14 (R&D Systems Inc., Minneapolis, MN). 100μl of assay diluent (RD1-43) was added to each well of a 96 well plate followed by 100 μl of assay diluent. This was incubated for 2 hours at room temperature. The lysis was then stopped adding 50 μl of stop solution (R&D Systems Inc., Minneapolis, MN). The optical density of the samples was measured in a spectrophotometer at a wavelength of 450 nm (DU-20 Beckam, Fullerton, CA, USA) (Appendix, Section C).

RT-PCR

RNA extraction was performed on the second series of samples according to Tryzol standard protocol (Appendix, Section D). For FGF-2 mRNA evaluation a reverse transcriptase (RT) reaction for cDNA (Invitrogen)

(Apendix, Section E) and a polymerase chain reaction (PCR) (Invitrogen) were carried out (Apendix, section F). GAPDH was run as house keeping gene and human dental pulp fibroblasts (HDPF) as controls. For semi-quantitative data analysis the mRNA expression was normalized to GAPDH and densitometric analysis by image-J was done. (Appendix, Section G).

Real-Time PCR

Following semi-quantification, Real Time PCR (Applied BioSystems) was carried for quantitative analysis. Reverse Transcriptase (RT) was preformed with 0.5µg total RNA and Oligo (dt) using SuperScripIII (Invitrogen. Carlsbad, CA, USA) in a 20µl reaction at 65 °C 5 min, 25 °C 5min, 55 °C 60 min and 70 °C 15min. Detection of FGF-2 and GAPDH expression level were performed with, 1µl of RT product and a 20 µl solution containing primers and probe from TaqMan Gene Expression Assays (GAPDH: S.O No: 185831521 and FGF-2: S.O No: 185859498) (Applied Biosystems, Foste City, CA, USA) were used. 30µl of PCR reactions were set with TaqMan Universal PCR Master Mix (Lot No: MP2182) (Applied Biosystems, Foste City, Ca, USA) manufactured by Roche prepared with 15 µl of master mix, 1.5 µl of primer, 3.5 µl of water and 8 µl of cDNA per well. Thermal cycling condition inlcuded 50°C 2 min, 95 °C 10 min followed by 40 cycles of 95°C 15 sec and 60 °C 1 min. Cycles were

performed using an ABI Prism Sequence Detectiion System 7700 (Applied Biosystems). The experiment was performed in triplicates and analyzed by standard curve method. (Appendix, Section H).

Statistical Analysis

A power analysis was conducted to calculate the minimum sample size required to accept the outcome. Based on a *t*-test with two-sided significance level of 0.05, with a sample size of n=32, a 90% power can be detected. Statistical analysis was performed on these data to test the proposed hypothesis by comparing the FGF-2 mRNA concentration between the group with deep caries, using One Way ANOVA or student t-test on Ranks with SIGMASTAT 2.0 statistical software (SPSS, Chicago, IL). The level of significance was determined at $P \leq 0.05$. For the Real-Time PCR samples were run in triplicates and analyzed by standard curve method.

3. RESULTS

The FGF-2 protein concentrations found in sound non-decayed teeth was from 13 pg/ml up to 928 pg/ml. Within the shallow decay group the lowest protein concentration ranged as low as 58 pg/ml and as high as 840 pg/ml. Within the deep decay group the lowest protein concentration ranged as low as 13 pg/ml and as high as 746 pg/ml. The distribution of the samples in the **shallow** decay group by sex was: 9 females and 6 males, by age: 21-55 y/o and tooth type 15 anterior teeth and 15 posterior teeth.. Optical density and FGF-2 protein concentration in pg/ml measured by ELISA. (Table 1).

The sample distribution in the **deep** decay group by sex was: 6 females and 9 males, by age: 25-54 y/o, and tooth type 7 anterior teeth and 23 posterior teeth. Optical density and FGF-2 protein concentration in pg/ml measured by ELISA. Table 2.

Table showing mRNA concentrations from control and decayed teeth prior to RT-PCR and Real-Time PCR analysis. Table 3.

FGF-2 Protein Expression.

FGF- 2 protein expression was evaluated by ELISA after standardization to 200 μg/ml per sample. When comparing FGF-2 expression in shallow and deep decayed groups with their non-decay controls no statistical significance was found ($P<0.942$ for shallow decay) and ($P<0.642$ for deep decay by t-test) (Figs: 5 and 9). In the shallow caries group, FGF-2 expression was up-regulated (increased) in 9 of 15 samples (60%) (Fig. 6 and 7) and down-regulated (decreased) in 6 of 15 samples (40%) (Fig. 6 and 8). In the deep caries group, FGF-2 expression was up-regulated in 7 of 15 samples (46%) (Figs: 10 and 11) and down-regulated in 8 of 15 samples (54%) (Figs: 10 and 12).

A statistical significant difference was noted within those samples in the shallow decay group showing up-regulation (P<000.6, *t*-test) and down-regulation (P<0.0035 *t*-test) in FGF-2 expression (Fig. 7 and 8). The same was also noted for those samples within the deep decay group showing up-regulation (P<0.0039 *t*-test) and down-regulation (P<0.0041 *t*-test) in FGF-2 expression (Figs: 11 and 12). A trend of FGF-2 up-regulation was noticed in the shallow decay group when analyzing the data of FGF-2 expression from the teeth that had up-regulation versus their controls (Fig. 5).

FGF-2 mRNA Expression

mRNA FGF-2 expression was evaluated by RT-PCR (Fig. 13). For semi-quantitative data analysis the mRNA expression was normalized to GAPDH by densitometric analysis. This analysis showed similar trends of increase or decrease of mRNA FGF-2 expression as compared to the expression analyzed by ELISA in 10 of 15 samples (67%) for the shallow decay group (Fig.14) and in 8 of 15 samples (54%) for the deep decay group (Fig. 15).

Real-Time PCR (qPCR)

Quantitative Real time mRNA FGF-2 expression did not show a statistically significant difference when comparing the non-carious group versus the carious groups (shallow and deep decay) (P<0.32). This analysis showed similar trends of increase in mRNA FGF-2 expression as analyzed by RT-PCR in 5 of 15 samples for the shallow decay group (#4 SD, #11 SD, #19 SD, #20 SD, #23 SD) and in 4 of 15 samples for the deep decay group (#9 DD, #17 DD, #21 DD, #30 DD). (Fig.16). SD: Shallow decay. DD: Deep decay.

Samples distribution for the **shallow decay** group and FGF-2 expression analyzed by ELISA

Sample	Sex	Age	Tooth type	Optical density	FGF-2 pg/ml
1.1 Control	F	28 y/o	Canine #27	0.9033	331.22
1.3 SD			Central #25	1.6514	656.48
2.1 Control	F	35 y/o	Premolar #20	1.2475	480.87
2.3 SD			Canine #6	0.8609	520.78
3.1 Control	M	38 y/o	Canine #22	1.6737	666.17
3.3 SD			Molar #19	1.1534	439.96
4.1 Control	M	53 y/o	Molar #30	0.1302	0.00
4.3 SD			Molar #14	0.4411	130.26
5.1 Control	M	55 y/o	Molar #31	0.7158	249.70
5.3 SD			Molar #14	1.061	399.78
6.1 Control	F	46 y/o	Canine #6	0.9859	367.13
6.3 SD			Central #8	1.4847	584.00
7.1 Control	F	38 yo	Premolar #13	0.9899	368.87
7.3 SD			Canine #22	0.2894	64.30
8.1 Control	F	41 y/o	Central #26	1.4312	560.74
8.3 SD			Central #24	0.8145	292.61
9.1 Control	F	47 y/o	Molar #15	2.278	928.91
9.3 SD			Premolar #28	1.0349	388.43
10.1 SD	F	39 y/o	Canine #11	1.0973	415.57
10.3 SD			Molar #30	1.5067	593.57
11.1 Control	F	36 y/o	Canine #27	1.6043	636.00
11.3 SD			Premolar #28	1.0344	388.22
12.1 Control	M	21 y/o	Central #24	0.3209	78.00
12.3 SD			Premolar #29	0.813	291.96
13.1 Control	F	24 y/o	Incisor #7	0.5943	196.87
13.3 SD			Incisor #8	0.2759	58.43
14.1 Control	M	32 y/o	Premolar #29	0.4063	0.0
14.3 SD			Molar #3	0.6669	154.9
15.1 Control	M	44 y/o	Central #26	0.8797	420.9
15.3 SD			Premolar #4	1.215	840.0

Table 1. Sample distribution in the shallow decay group by sex, age, and tooth type. Optical density and FGF-2 protein concentration in pg/ml measured by ELISA.

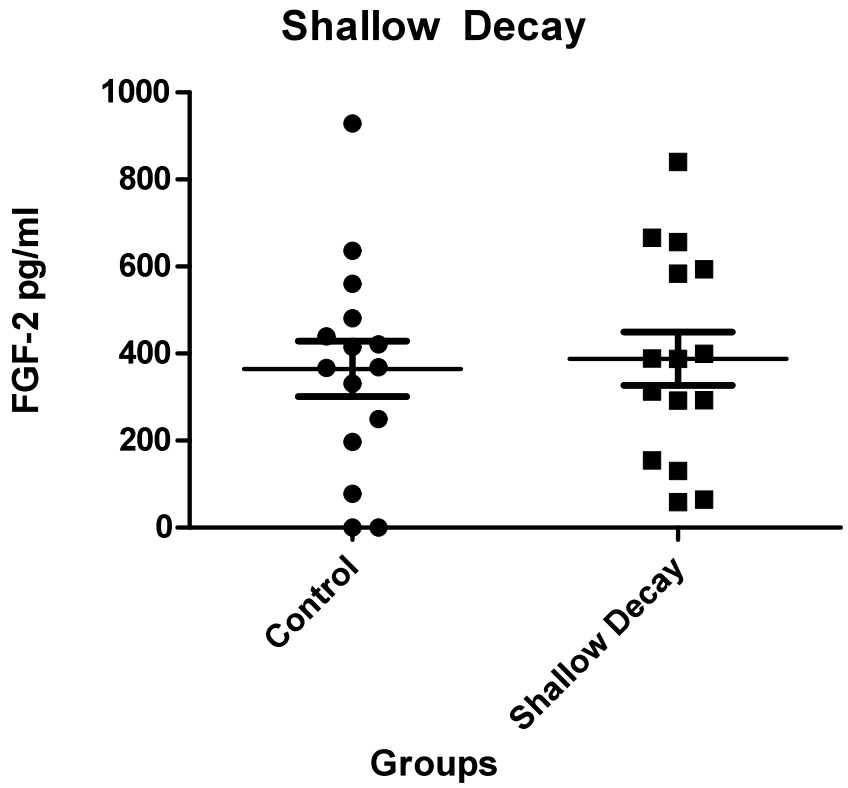


Figure 5. FGF-2 protein expression showing the general trend and range of expression for the shallow decay group. (P<0.942) (*t*-test).

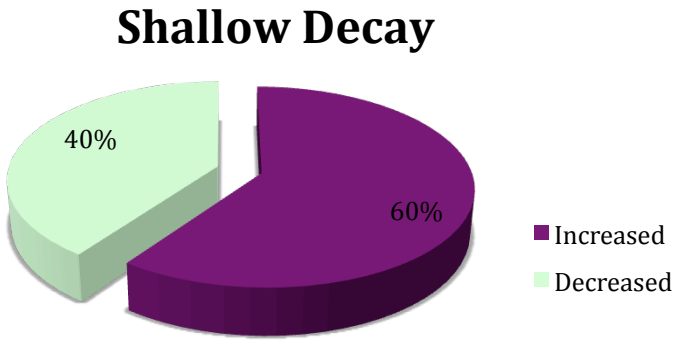


Figure 6. Depicting percentage of samples showing FGF-2 expression up-regulated in 9 of 15 samples (60%) and down-regulated in 6 of 15 samples (40%).

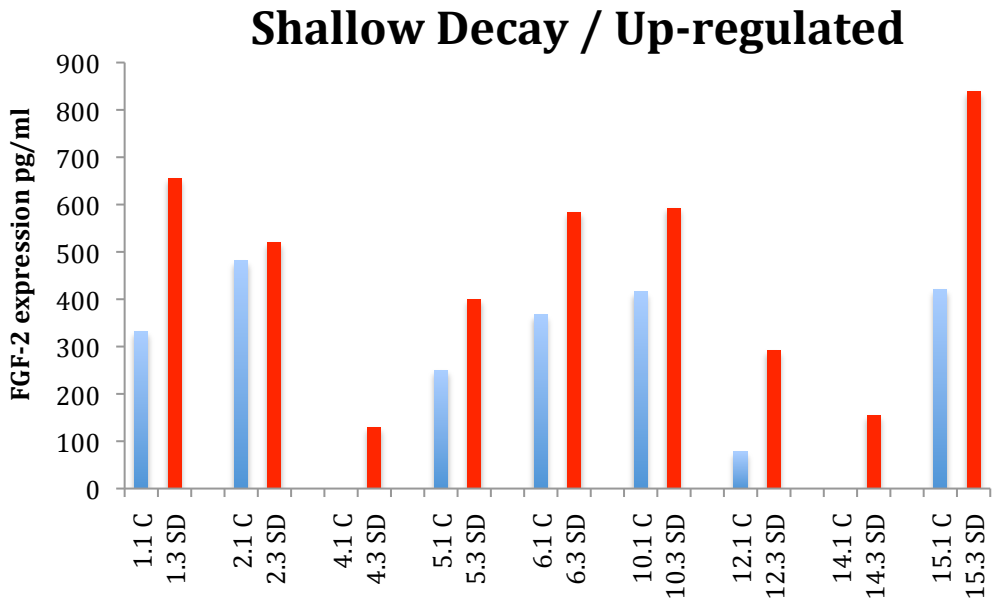


Figure 7. Samples within shallow decay group showing **up-regulation** of FGF-2 protein expression. A statistical significant difference was present between each control and decayed sample (P<000.6) (*t*-test). C: Non-decayed tooth. SD:shallow decay tooth.

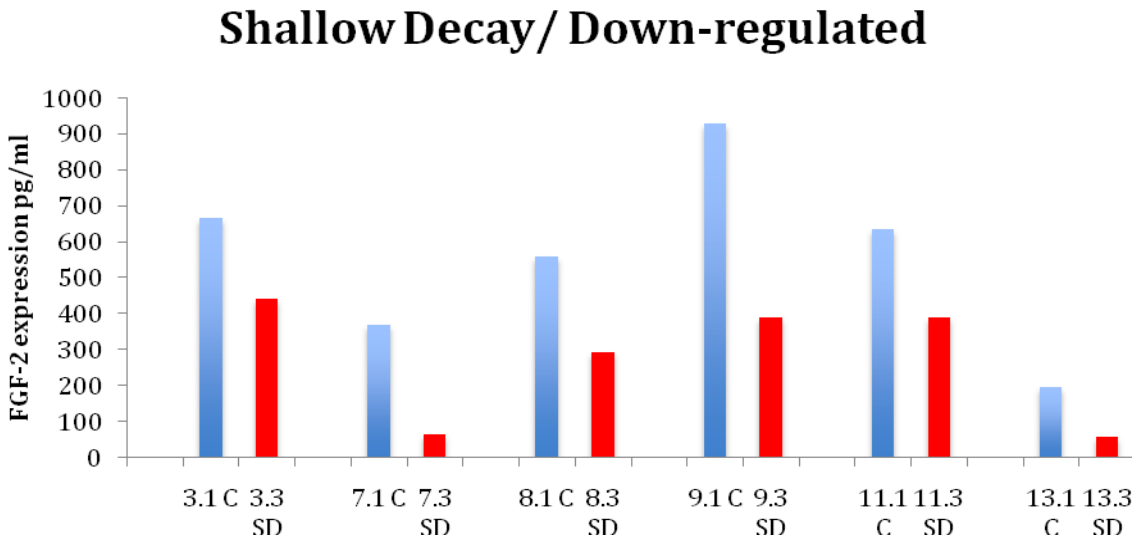


Figure 8. Samples within shallow decay group showing **down-regulation** of FGF-2 protein expression. A statistical significant difference was present between each control and decayed sample (P<0.0035) (*t*-test). C: Non-decayed tooth. SD:shallow decay tooth

Samples distribution for the **deep decay** group and FGF-2 expression analyzed by ELISA

Sample	Sex	Age	Tooth type	Optical Dens	pg/ml
1.1 Control	F	54 y/o	Canine #11	0.1724	13.43
1.3 DD			Molar #2	0.5767	189.22
2.1 Control	F	43 y/o	Incisor #26	0.5242	166.39
2.3 DD			Premolar #29	0.2499	47.13
3.1 Control	F	42 y/o	Premolar #21	0.8655	314.78
3.3 DD			Premolar #5	0.1762	15.09
4.1 Control	F	48 y/o	Premolar #13	0.9816	365.26
4.3 DD			Molar #3	0.7789	277.13
5.1 Control	M	38 y/o	Incisor #9	0.2711	56.35
5.3 DD			Premolar #4	0.3373	85.13
6.1 Control	M	29 y/o	Premolar #21	0.505	158.04
6.3 DD			Molar #18	0.0775	0.00
7.1 Control	M	27 y/o	Premolar #29	0.996	371.52
7.3 DD			Molar #30	1.8574	746.04
8.1 Control	M	44 y/o	Premolar #12	0.3298	81.87
8.3 DD			Molar #14	0.0946	0.00
9.1 Control	M	25 y/o	Molar #19	0.3618	95.78
9.3 DD			Canine #6	0.1191	0.00
10.1 Control	F	36 y/o	Premolar #21	1.5889	629.30
10.3 DD			Incisor #7	0.9551	353.74
11.1 Control	M	53 y/o	Molar #18	0.2166	32.65
11.3 DD			Molar #19	0.9225	339.57
12.1 Control	F	39 y/o	Canine #11	0.3062	71.61
12.3 DD			Molar #30	0.7076	246.13
13.1 Control	M	34 y/o	Premolar #4	0.4636	140.04
13.3 DD			Canine #6	0.4044	114.30
14.1 Control	M	37 y/o	Molar #18	0.2716	56.57
14.3 DD			Molar #3	0.5582	181.17
15.1 Control	M	30 y/o	Molar #19	0.2904	64.74
15.3 DD			Premolar #4	0.719	251.09

Table 2. Sample distribution in the deep decay group by sex, age, and tooth type. Optical density and FGF-2 protein concentration in pg/ml measured by ELISA.

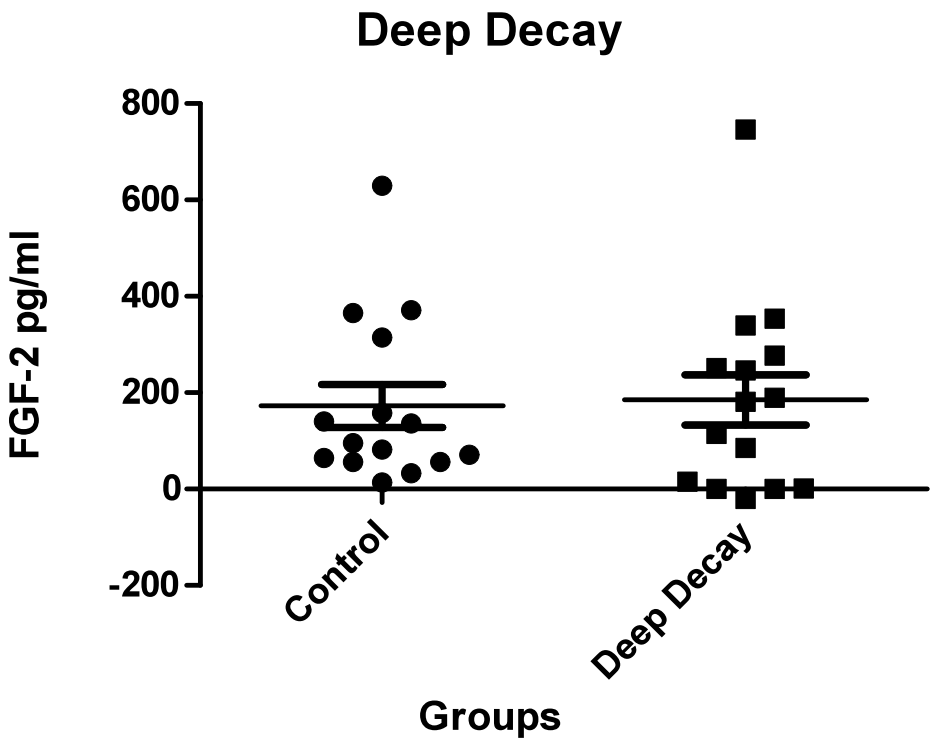


Figure 9. FGF-2 protein expression levels showing the general trend and range of expression for the deep decay group. (P<0.642) (t-test).

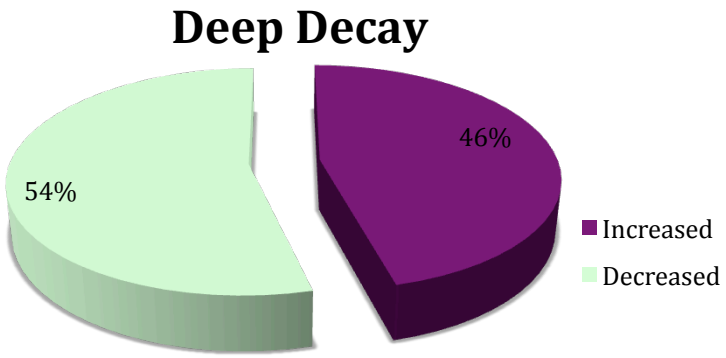


Figure 10. Depicting percentage of sample showing FGF-2 expression being up-regulated in 7 of 15 samples (46%) and down-regulated in 8 of 15 (54%).

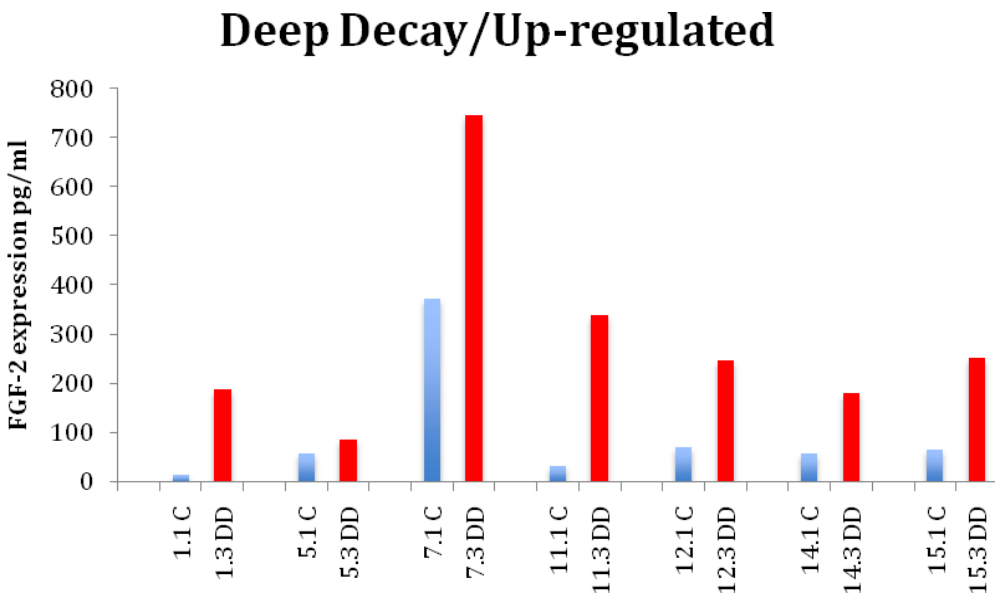


Figure 11. Samples within deep decay group showing **up-regulation** of FGF-2 protein expression. A statistical significant difference was present between each control and decayed sample (P<0.0039) (*t*-test). C: Non-decayed tooth. DD:deep decay tooth.

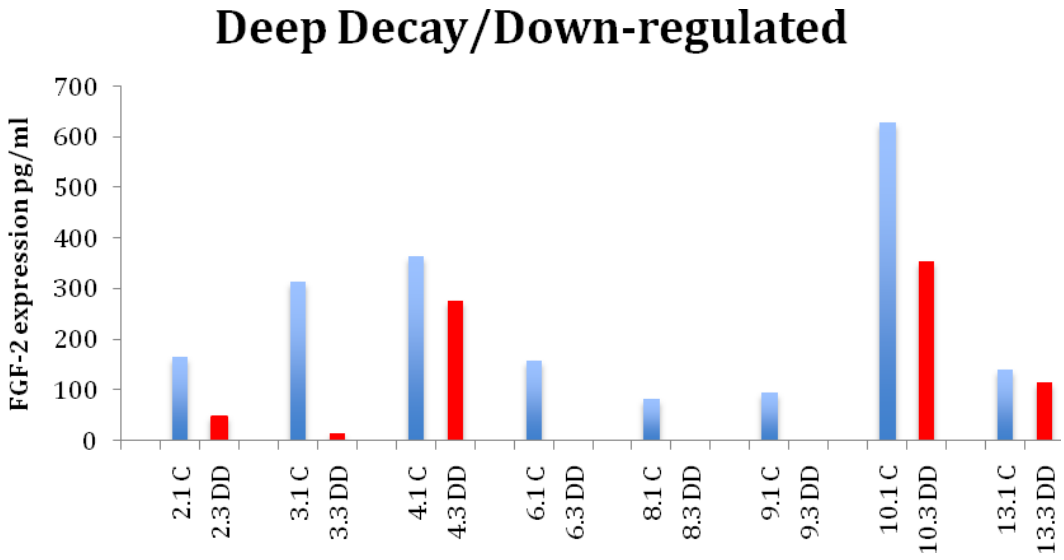


Figure 12. Samples within deep decay group showing **down-regulation** of FGF-2 protein expression. A statistical significant difference was present between each control and decayed sample (P<0.0041) (*t*-test). C: Non-decayed tooth. DD: deep decay tooth.

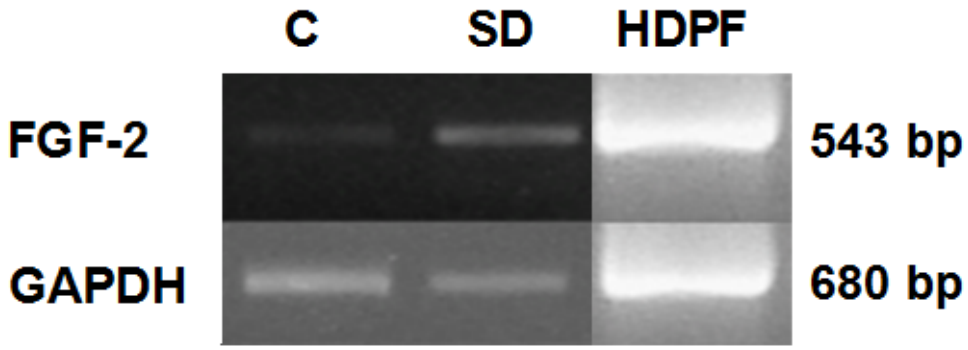
mRNA Concentrations

	Control	Decay
Patient 1	0.3420 μg/ml	1.0703 μg/ml
Patient 2	0.5990 μg/ml	0.4984 μg/ml
Patient 3	1.0687 μg/ml	0.5313 μg/ml
Patient 4	0.2439 μg/ml	0.4162 μg/ml
Patient 5	0.4783 μg/ml	0.7769 μg/ml
Patient 6	1.0582 μg/ml	0.4419 μg/ml
Patient 7	0.2425 μg/ml	0.4542 μg/ml
Patient 8	0.4852 μg/ml	0.5336 μg/ml
Patient 9	0.4029 μg/ml	0.5661 μg/ml
Patient 10	0.5843 μg/ml	0.5460 μg/ml
Patient 11	0.4495 μg/ml	3.0871 μg/ml
Patient 12	0.9951 μg/ml	1.2436 μg/ml
Patient 13	2.2134 μg/ml	1.0184 μg/ml
Patient 14	2.5854 μg/ml	1.1539 μg/ml
Patient 15	1.3756 μg/ml	0.9344 μg/ml
Patient 16	1.6111 μg/ml	0.8942 μg/ml
Patient 17	1.5854 μg/ml	2.0730 μg/ml
Patient 18	1.8864 μg/ml	1.0944 μg/ml
Patient 19	0.6588 μg/ml	4.0554 μg/ml
Patient 20	1.1702 μg/ml	3.0542 μg/ml
Patient 21	0.7277 μg/ml	1.4903 μg/ml
Patient 22	2.6693 μg/ml	1.6942 μg/ml
Patient 23	0.5278 μg/ml	1.6650 μg/ml
Patient 24	2.1949 μg/ml	0.5964 μg/ml
Patient 25	1.4859 μg/ml	1.3918 μg/ml
Patient 26	1.8708 μg/ml	2.3307 μg/ml
Patient 27	1.6435 μg/ml	2.5361 μg/ml
Patient 28	1.3854 μg/ml	1.0382 μg/ml
Patient 29	2.5787 μg/ml	2.4038 μg/ml
Patient 30	1.8171 μg/ml	1.3555 μg/ml

Table 3. Table showing mRNA concentration from control and decayed teeth prior to RT-PCR and Real-Time PCR analysis.

mRNA FGF-2 Expression analyzed by RT-PCR.

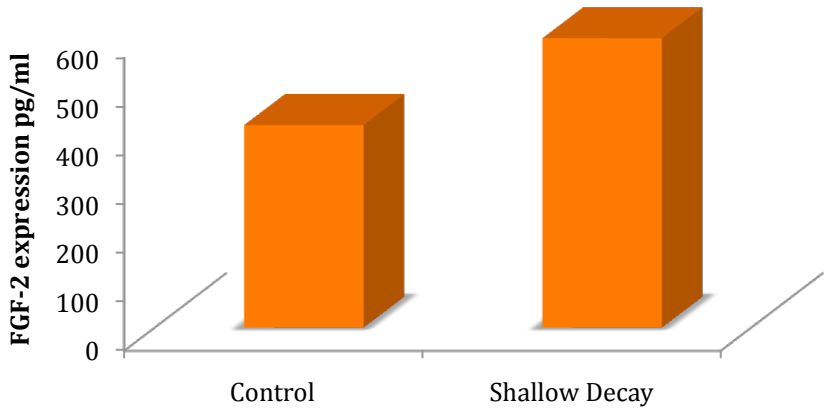
A. Sample #10 / Shallow Decay group



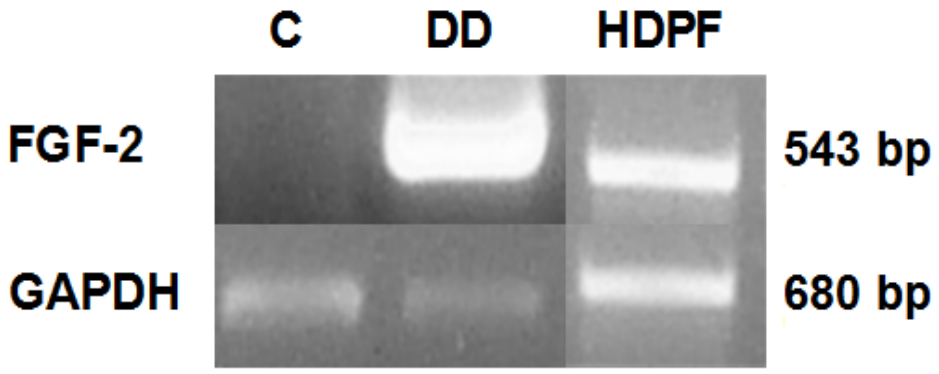
Densitometry/FGF-2: 1283 (C) 3276 (SD)

Densitometry/GAPDH: 1876 (C) 973 (SD)

ELISA



B. Sample #12 / Deep Decay group



Densitometry/FGF-2: 785 (C) 3411 (DD)

Densitometry/GAPDH: 1963 (C) 1061 (DD)

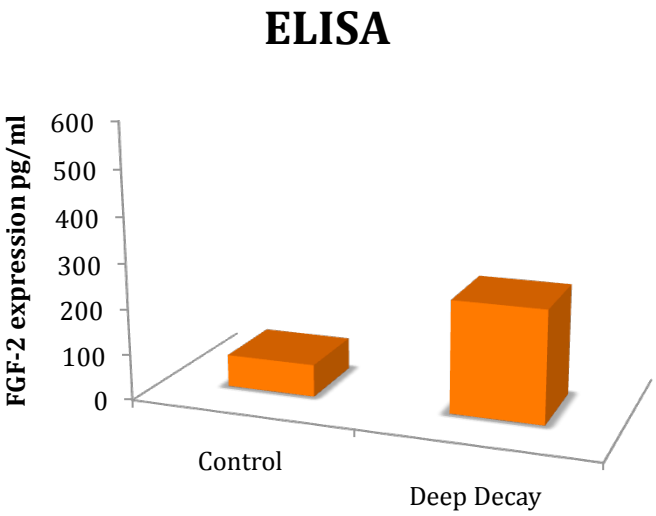


Figure 13. Selected samples from shallow decay (SD) (sample #10, A), and deep decay groups (DD) (sample #12, B) showing up-regulated FGF-2 mRNA and protein expression. mRNA FGF-2 expression as analyzed by RT-PCR. GAPDH was run as house keeping gene and Human dental pulp fibroblasts (HDPF) as controls. FGF-2 protein expression as analyzed by ELISA.

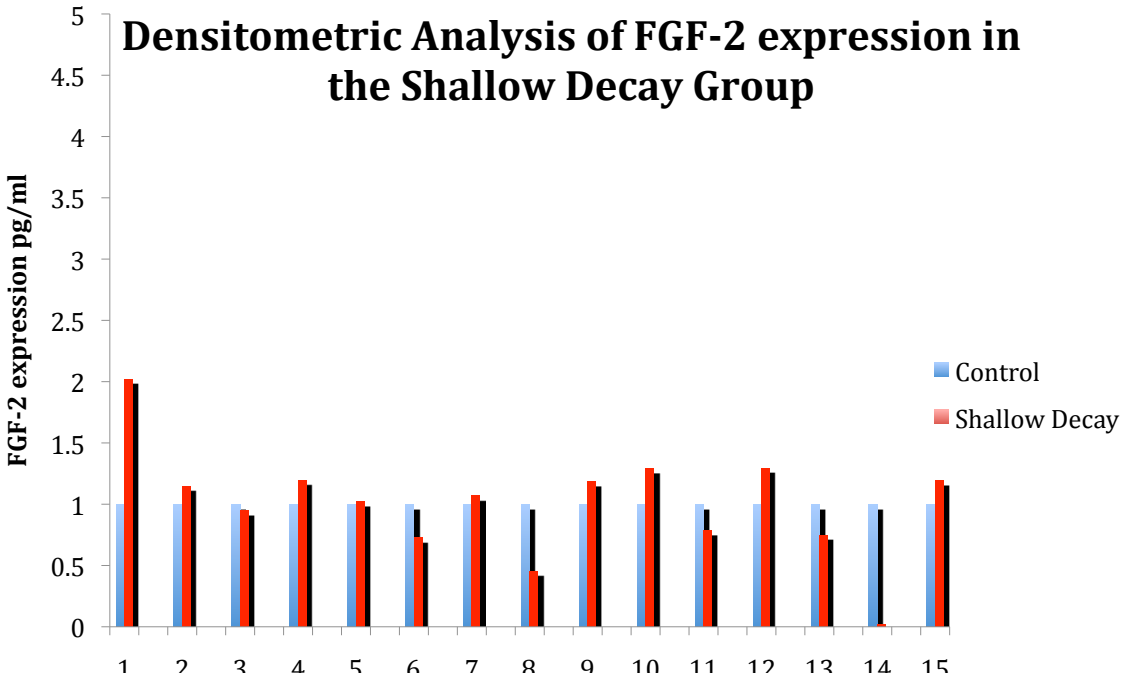


Figure 14. FGF-2 expression in the shallow decay group showing up-regulation and down-regulation in the control vs the shallow decay group. The optical density of each amplified band was calculated using the Image J software Program (1.32 j) and then numerically expressed as the relative density and normalized to the house keeping gene (GAPDH).

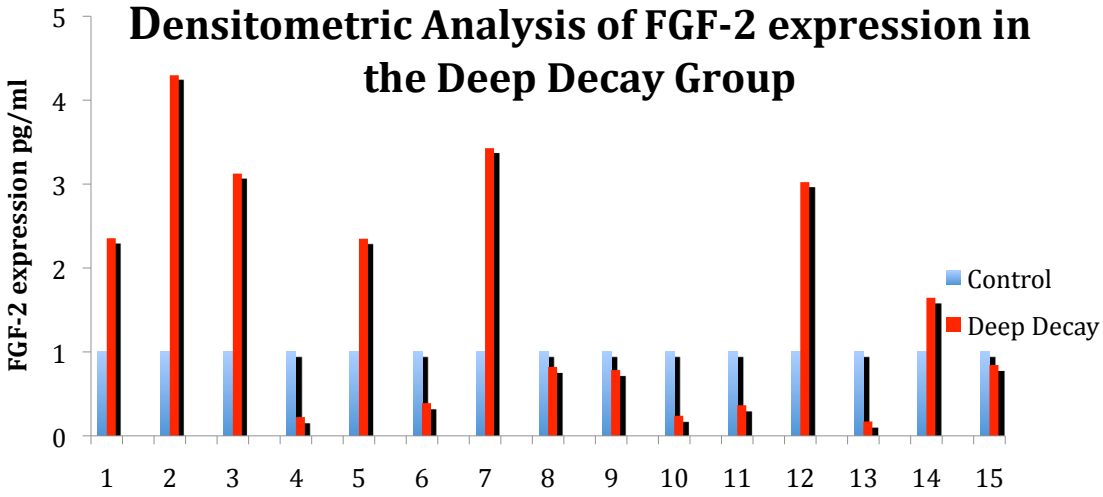


Figure 15. FGF-2 expression in the deep decay group showing up-regulation and down-regulation in the non-decay vs the deep decay teeth. The optical density of each amplified band was calculated using the Image J software Program (1.32 j) and then numerically expressed as the relative density and normalized to the house keeping gene (GAPDH).

Real-Time PCR (qPCR)

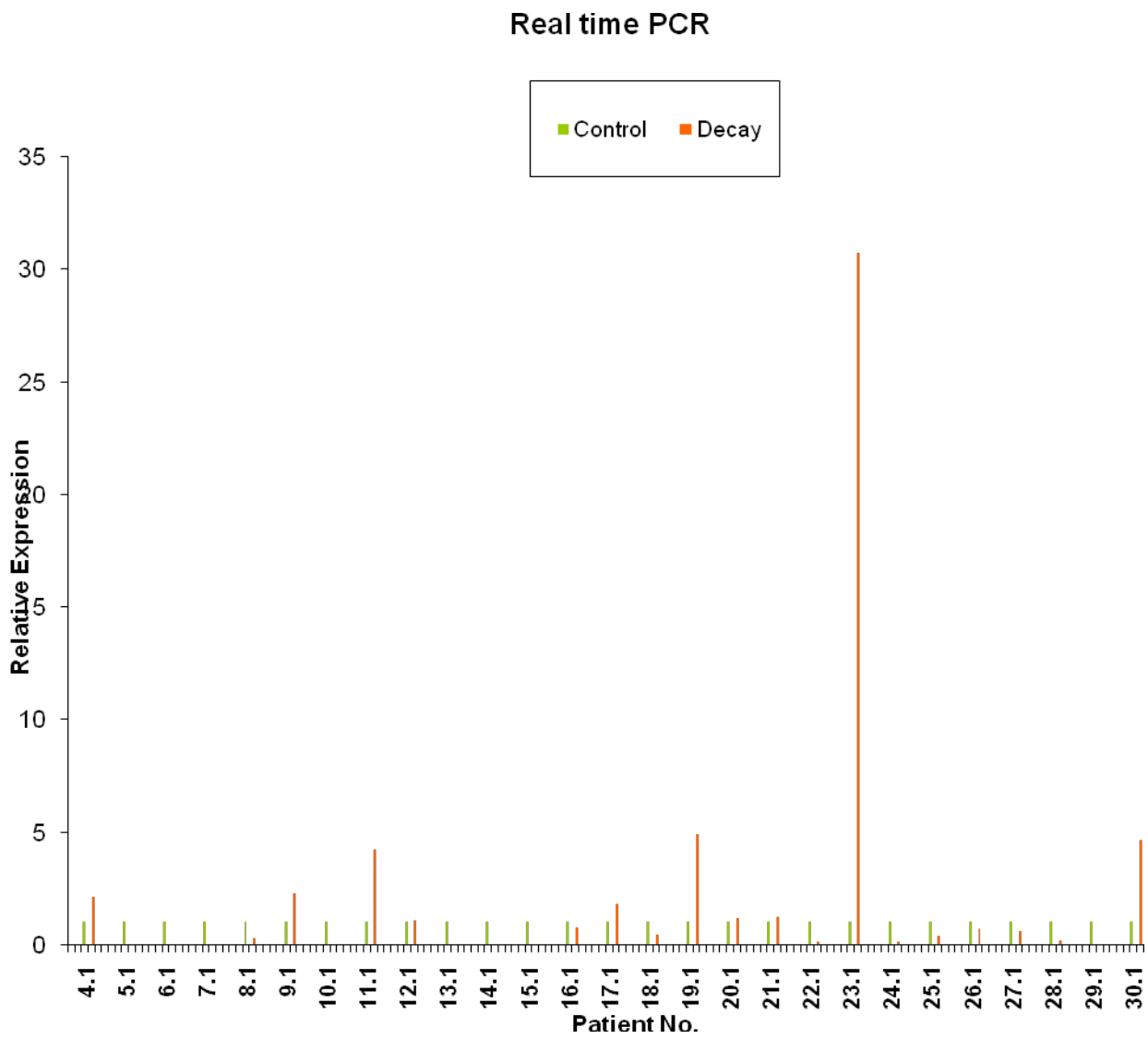


Figure 16. Graph representing the relative expression by means of Real-Time PCR. Quantitative Real time mRNA FGF-2 expression did not show a statistically significant difference when comparing the non-carious group versus the carious groups. ($P<0.32$).

Summary of the Results

The analysis of protein expression evaluated by ELISA showed up-regulation in the shallow caries group in 9 of 15 samples (60%) and down-regulated in 6 of 15 samples (40%). In the deep caries group FGF-2 expression was up-regulated in 7 of 15 samples (46%) and down-regulated in 8 of 15 samples (54%). No statistical significant difference was found when comparing FGF-2 expression in all shallow or deep decayed samples to non-decayed samples. Densitometric analysis of RT-PCR showed similar trends of increase or decrease of FGF-2 expression as analyzed by ELISA in 10 of 15 (67%) in the shallow decay group and 8 of 15 (54%) in the deep decay group. Quantitative analysis measured by Real Time PCR did not show a statistically significant difference when comparing FGF-2 expression in the non-carious versus the carious group. Age, sex and tooth type did not appear to be determinant factors in the up-regulation or down-regulation of FGF-2 within the shallow decay or deep decay group

4. DISCUSSION

The dental pulp has an inherent regenerative potential. Angiogenesis is critical in healing and is regulated by the interplay of numerous growth factors. FGF-2 is one of these growth factors and plays a significant role in the revascularization of damaged or traumatized tissue (Gerwins *et al.,* 2000). This study demonstrates that human pulp tissue contains fibroblast FGF-2 both in health and during inflammation.

A number of studies have reported the release of FGF-2 from normal (Derringer *et al.,* 2004, Shimabukuro et al., 2005, Hung *et al.,* 2006, and Hung *et al.,* 2007) and injured human pulp tissue (Hung *et al.,* in 2007). In the last mentioned study fibroblasts were cultured in vitro and mechanically injured with scalpels. The production of this growth factor during inflammation has not been investigated.

In the current study FGF-2 concentration was measured in sound and decayed teeth by ELISA. Up-regulation of FGF-2 was more common in the shallow than in the deep decay group although the difference was not statistically significant. The same pattern was found when mRNA levels for FGF-2 were evaluated by RT-PCR. The hypothesis that pulp tissue removed from teeth with carious lesions would show an increase in FGF-2 expression over tissue from intact teeth is thus not supported. This is despite the fact

that FGF-2 is present in dentin and would be released during the breakdown due to caries (Goldberg and Smith, 2004).

The failure to support the hypothesis could be due to a number of factors. The literature supports how the presence of bacteria in carious pulp exposure may modify growth factor release and cell activity (Rutherford *et al.,* 2000; Rutherford *et al.,* 2001). It has been shown how bacteria that invade the dentin can diffuse through dentinal tubules and become involved in the pathogenesis of pulpitis (Love and Jenkins, 2002). Depth of bacterial invasion may depend, at least in part, upon tubule diameter, since this determines the rate of solute diffusion (Pashley, 1992). Sclerotic or obliterated tubules will physically impede bacterial invasion and can result in regional differences in bacterial invasion of dentin. Limiting nutritional supply may influence the depth of bacterial penetration. This may account for the higher numbers of cariogenic bacteria within superficial dentin (Edwardsson, 1987), where the presence of fermentable carbohydrates and oxygen from the oral cavity is likely to be higher than in deeper dentin. Collected decayed teeth in our study were grouped into shallow or deep decay based on the depth of decay and its proximity to the pulp chamber. This was determined by clinical observation after vertical sectioning of the teeth. Histologic evaluation to determine the exact depth of bacterial

invasion and type of cariogenic microflora present in our samples was not determined. This is a limitation of our study. It would have been relevant to measure the FGF-2 expression within these sections of decayed dentin to determine if any correlation exists between depth of bacterial invasion and growth factor expression. Perhaps this could have provided clarification for the trend of up-regulation present within the shallow decay group and not the deep decay group.

It has been shown how the distribution of growth factors between the soluble and insoluble tissue compartments of the dentin matrix may reflect the manner in which they become sequestered within the tissue and will also determine their potential release characteristics (Clarks and Smith, 2000). Transforming growth factors (TGFs) are reportedly associated with several molecules in dentin matrix (Smith *et al.,* 1998) and their distribution between the soluble and insoluble tissue compartments would determine the sites of growth-factor localization. The observed distribution of angiogenic growth factors in dentin presumably reflects their association with various extracellular matrix molecules. This association may influence not only their distribution but also their biological effects, as interactions with extracellular matrix molecules provide one level of regulation of growth-factor activity (Gospodarowicz *et al.,* 1990; Kim *et al.,* 1998). The higher concentrations

of most of these growth factors in the soluble tissue compartment will allow more ready release during carious or other injuries. Both growth factor distribution and concentration between these dentin matrix compartments are factors that may have influenced the difference in FGF-2 expression within our samples.

Before actual caries exposure, the dental pulp beneath shallow caries is capable of mounting innate immune responses to slow down the caries invasion. The ability of the odontoblast to respond to caries or injury and up-regulate its secretory activity leading to deposition of reactionary dentin is well-established (Smith *et al.,* 1994). The process of reactionary dentinogenesis involves up-regulation of odontoblast activity, often in quiescent cells at the stage of physiological secondary dentinogenesis, in response to the injury stimulus (Smith *et al.,* 1995). The nature of the signaling process from this stimulus may be rather variable and has been hypothesized to result from the release of growth factors and other bioactive molecules from the dentin matrix during injury (Smith *et al.,* 1995). Consequently, the up-regulatory signaling may be rather non-physiological and lead to compositional differences in matrix secretion during dentinogenesis. This may have played a role in the signaling for the up-regulation of secretory activity seen in the FGF-2 expression in the shallow

decay group. Thus, allowing a trend of up-regulation within the shallow decay teeth.

Morphological changes in odontoblast beneath caries lesions have been reported (Bjørndal and Darvann, 1998), and in very active or deep lesions, tertiary dentinogenic processes may be absent altogether (Bjørndal and Darvann, 1998). Cell signaling in our samples with more severe pulp injuries may have down-regulated the secretory activity showing a decrease in FGF-2 expression within the deep decay group. Furthermore, these samples with deep decay may have witnessed a more chronic stage of inflammation with possible partial necrosis, which could have decreased the FGF-2 secretion within the pulp.

Wound healing is a complex process involving inflammation. FGF-2 is considered to participate in an early stage of the wound-healing process (Gibran *et al.,* 1994; Yu *et al.,* 1994). It has been reported that during the early stages of wound healing FGF-2 accelerates this process by increasing inflammatory cell infiltration (polymorphonuclear leukocyte, monocyte, and macrophage) into the wound (Tanaka *et al.,* 1996). This may provide an explanation for the trend of FGF-2 up-regulation present in the shallow decay group, which was probably undergoing more of an acute inflammatory phase, thus, an increase in FGF-2 participation. If this

hypothesis is true, FGF-2 down-regulation would be expected in those teeth within the deep decay group which were probably undergoing a more chronic stage of inflammation, as noted within our results.

A particular observation to be made is that FGF-2 expression in the coronal pulp could differ from the radicular pulp. The coronal pulp is larger and contains many more elements than radicular pulp. Both pulp areas contain the same elements, although the cells, fibers, blood vessels, and nerves are more numerous in the coronal pulp (Okihi *et al.,* 1997). The distribution of sympathetic fibers is also highest in blood vessels in the pulp horns near the odontoblastic region and lowest in the apical region (Avery *et al.,* 1980). The cell-rich zone is present in the coronal pulp and has a relatively high density of cells. This zone is discernible due to its higher density of fibroblast than the pulp proper and is much more prominent in the coronal pulp than in the radicular pulp. After each tooth was sectioned the pulp was removed and cut into halves. Providing sample analysis from coronal and radicular pulp. It would be reasonable to speculate that the cellular differences between these two sections could have influenced the expression of FGF-2. We hypothesize that a higher concentration of FGF-2 would probably be detected coronally than apically.

Additionally, the nature of the signaling process may be variable between individuals. Although both decayed and non-decayed teeth were obtained from the same patient, biological and morphological differences in patients can give rise to inter-patient and intra-patient variation in response (Derringer *et al.,* 2004). As well as alteration in levels and response to FGF-2 throughout life (Gao *et al.,* 1996), there is also evidence that different individuals may possess different levels of angiogenic growth factors. Large variations in levels of VEGF have been reported in periodontal tissues with age and inflammatory factors (Booth *et al.,* 1998). Similarly variations in levels of FGF-2 in the pulp during inflammation may occur.

A power analysis was conducted to calculate the minimum sample size required to accept the outcome. Based on a *t*-test with two-sided significance level of 0.05, with a sample size of n=32, a 90% power could be detected. A total of 30 matched pairs were collected in our study due to the difficulty in obtaining a decayed and a non-decayed tooth from the same patient. It would be interesting to evaluate the results of FGF-2 expression with a larger sample size and determine if any difference in growth factor expression would be present.

Age, sex and tooth type within our samples were not explanatory variables for the up-regulation or down-regulation of FGF-2. Although comparison with similar studies would be interesting, there are, however, no studies evaluating the expression of FGF-2 in human inflamed pulp tissue to compare with our results.

The hypothesis is worthy of further investigation and the observations stated above should be considered. This investigation brings more questions regarding FGF-2 expression in the pulps of teeth with carious lesions. Future studies demonstrating and quantifying angiogenic growth factor expression in pulp tissue should assess the same in the carious dentin of the same specimen. Correlation in growth factor expression between dentin and pulp could provide further understanding of cell-signaling mechanisms. Studies that exploit cell-signaling properties could have potential for the development of therapeutic strategies to modulate growth factor expression during pulpitis and/or dentinogenesis.

This study contributes to our understanding of the pathophysiology of the dental pulp and the mechanisms responsible for tooth pulp repair. It opens another research orientation concerning angiogenic growth factor expression and their capabilities for changing the clinical management of disease in the dentin-pulp complex.

5. CONCLUSIONS:

1. Human pulp tissue contains fibroblast growth factor (FGF-2) both in health and during inflammation.
2. In the shallow caries group FGF-2 expression was up-regulated in 9 of 15 samples (60%) and down-regulated in 6 of 15 samples (40%).
3. In the deep caries group FGF-2 expression was up-regulated in 7 of 15 samples (46%) and down-regulated in 8 of 15 samples (54%).
4. No statistical significant difference was found when comparing FGF-2 expression in shallow and deep decayed groups with their non-decay controls.
5. A trend of FGF-2 up-regulation was noticed in the shallow decay group when analyzing the data of FGF-2 expression from the teeth that had up-regulation versus their controls.
6. Densitometric analysis of RT-PCR showed similar trends of increase or decrease of FGF-2 expression as analyzed by ELISA in 10 of 15 (67%) in the shallow decay group and 8 of 15 (54%) in the deep decay group.
7. Quantitative analysis measured by Real Time PCR did not show a statistically significant difference when comparing FGF-2 expression in the non-carious versus the carious group.
8. Age, sex and tooth type were not related to the expression of FGF-2 within either control nor carious group.

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APPENDIX

LABORATORY PROTOCOLS

A. Preparation of Specimens

The tooth surface was disinfected using a sterile gauze swab soaked in 70% ethanol. Tissue and deposits adhering to the outside of the tooth was removed with a sterile curette (Miltex 7-540 / G11-12).

Each tooth was affixed to a wooden block (3 cm x 3 cm x 1.5 cm) with Super Glue (Elmers Products, Columbus, OH). These blocks were then mounted on an Isomet Low Speed circular saw (Model 650, South Bay Technology, Inc., San Clemente, CA) (Fig. 3). The teeth were sectioned vertically with a lapidary blade 303 Series (MK-303 Professional, MK Diamond Products Inc., Calais, ME) while spraying sterile phosphate buffered saline (PBS-1X; Gibco) as a coolant. The saw was washed with 70% ethanol and sterile PBS before sectioning of the teeth.

After each tooth was sectioned the pulp removed intact using sterile blunt instruments. The harvested pulp was then vertically cut into halves. Each half was placed in a labeled eppendorf tube. Half of the tubes contained lysis buffer for protein (NP-40 Reagent/ Invitrogen). The rest of the tubes contained RNA lysis buffer (Trizol Reagent/ Invitrogen). All specimens were then stored at -80 ºC.

B. Preparation of Protein Lysates (NP-40 Lysis buffer)

1. Pulp tissue was thawed on ice.

2. Tissue was crushed (homogenized) with sterile pestles /Time: 20-30 min on ice.

3. Vortex for 20-30 sec every 3-5 min.

4. Spin 10 min at 13000 rpm, 4 ºC.

8. Collect the supernatant (protein lysate) and store in -80 ºC.

9. Reading of protein concentration by Bio-rad Protein (BIO-RAD Laboratories, Inc., Hercules, CA) Optical density measured at 595 nm in Spectrophotometer (GENios RECAN. Spectra Fluor Plus. M-Code: 1560063).

10. Protein concentration was standardized to 200 Ug/ml of total protein from each sample.

C. FGF-2 ELISA Protocol For Cell Culture Supernate Samples (R & D Systems Inc, Minneapolis, MN).

1. FGF-2 Standard Preparation: Reconstitute the FGF-2 Standard with 2 ml of the Calibrator Diluent RD5-14. This produces a stock solution of 640 pg/ml. Allow the standard to sit for 15 mins with gentle agitation prior to making diluents.

2. Standard Dilution Series: Using eppendorfs pipette 500 µl of Calibrator Diluent RD5-14 into each tube. Use the stock solution to produce a dilution series: (640 pg/ml, 320 pg/ml, 160 pg/ml, 80 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml).

3. Assay Procedure:

a Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal. Make sure to allow for triplication of each sample in the wells.

b. Add 100 µl of Assay Diluent (RD1-43) to each well.

c. Add 100 µl of Standard or sample to each well. Total protein concentration from each sample was previously standardized to 200 µg/ml and adjusted with the RD1-43 diluent. Cover with the adhesive strip provided and incubate for 2 hours at RT. Place the plate into the foil pouch

and seal it. Record the sample triplication on plate layout sheet provided.

d. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (approx 400 µl per wash), using a finnpipette (Fisher*brand*). Tap the well plate, gently on the bench to obtain complete removal of wash buffer, after the last wash. Wash buffer remnants will cause dilution of the assay reagents to be used next, interfering with the reaction giving erroneous results.

e. Add 200 µl of FGF-2 Conjugate to each well. Cover with an adhesive strip. Incubate for 2 hours at RT and place the plate into the foil pouch.

f. Repeat the washing as in step d, being careful to wash correctly so as to remove the unbound antibody and wash buffer completely.

g. In a dark room add 200 µl of Substrate Solution (A,100 µl plus B, 100 µl) to each well. Incubate for approximately 30 mins at RT.

h. Add 50 µl of Stop Solution to each well, observing a color change in each well. If the color change does not appear uniform gently tap the plate to ensure through mixing.

i. Determine the optical density of each well within 30 mins following step h, using a microplate reader set to 450 nm.

7. Re-calculate the concentration of growth factors in the medium by multiplying each sample reading from the microplate reader by a factor of

100, to allow for dilution (step 2). The standard preparation must be in pg/ml not ng/ml to prevent overflow, as pg/ml concentration is the standard which the microplate reader will use to compare to the triplicated samples.

8. Plot an excell graph using the database of the standard FGF-2 protein expression.

D. RNA EXTRACTION

1. Thawed, passed to eppendorf tubes from -80 ºC on ice (tissue was crushed) and keep at room temperature for 5 minutes.

2. Add chloroform (200 µl).

3. Shake by hand and keep at room temperature for 2-3 minutes.

4. Spin at 12.000 xg / 15 min/ at 4 ºC.

5. Transfer only aqueous phase to a new labeled eppedorf tube.

6. Add 0.5 ml of Isopropyl alcohol.

7. Kept at temperature for 10 min.

8. Spin at 12.000 xg/ 10 min/ 4 ºC.

9. Remove supernatant and wash pellet w/ 75% ethol in DNase water.

10. Spin at 7500 xg/ 5 min/ at 4 ºC.

11. Remove supernatant and re-suspend.

12. Measure mRNA with 1µl of sample on 99 of water (µg/ml).

13. Standardized to 0.1 µg/ml and stored at -80 ºC.

E. REVERSE TRANSCRIPTASE / First Strand cDNA Synthesis

Sequence of Primers:

FGF-2: Sense: 5'-GCC-GTC-AAG-GCC-CAC-CCT-G-3'
Antisense: 5'-ATG-CGC-AGG-AAG-AAG-CCC-CC-3'

GAPDH: Sense: 5' -GAC-CCC-TTC-ATT-GAC-CTC-AAC-T-3'
Antisense: 5' -CAC-CAC-CTT-CTT-GAT-GTC-ATC-3

1. 1 µl Oligo DT (200-500 mg). Random Primer (50-250 mg).
2. (0.5 µg mRNA).
3. 1 µl / dNTP.
4. DNase RNase Water up to 9 µl. Asjudted to final volume to 13 µl.

Mastercycler Gradient (Eppendorf Scientific Inc., New York, USA)

Steps : Program (TBRT): 65 C / 5 min

Hold at 4 ºC.

4 µl 5X First-Strand Buffer

1 µl 0.1M DTT

1 µl RNase OUT

1 µl SuperScript enzyme

Total Volume: 20 µl

Steps: Program (TBRT2): 25 ºC / 5 min

55 ºC / 60 min

70 ºC /15 min

Hold at 4 ºC.

Keep at -20 ºC. Dilute 20 µl in 80 µl (5X dilution) with DNase water prior to polymerase chain reaction (PCR).

F. Polymerase Chain Reaction (PCR)

Cocktail:

1. Add Water----------35.8 + 4 µl
2. 10x PCR Buffer----------5 µl + 1 µl
3. 50 mM MgCL2----------2 µl
4. 10 mM dNTP----------1 µl
5. Sense Primer (25mM)----------1µl
6. AntiSense Primer----------1µl
7. Taq Platinum Polymerse-------0.4 µl (Invitrogen Inc. 500 rxn (5µ/µl).

Total Volume per tube----------50 µl

8.cDNA----------6 µl

Mastercycler Gradient (Eppendorf Scientific Inc., New York, USA)

Steps: Program (ZZCPCR1):

1. 94 ºC.: 2 min

2. 94 ºC.: 30 sec

3. 55 ºC.: 30 sec

4. 72 ºC.: 30 sec

5. 72 ºC.: 10 min

6. 4 ºC.: Hold

Keep samples at -20 ºC.

G. DENSITOMETRIC ANALYSIS

1. PCR band images from TIFF files were transferred to Image J program (software Program 1.32 j).

2. Each band image pixel value was then individually analyzed. Distance and angles were measured and density histrograms created.

3. The optical density of each amplified band was calculated and then numerically expressed as the relative density and normalized to the house-keeping gene (GAPDH).

4. Graph representation was done via Excel program.

H. Real-Time PCR (qPCR)

Sequence of Probes:

GAPDH: Assay # Hs 99999905_m1
TGGGCGCCTGGTCACCAGGGCTGCT

FGF-2: Assay # Hs 00266645_m1
GAGCGACCCTCACATCAAGCTACAA

1. RT was preformed with 0.5µg total RNA and Oligo (dt) using SuperScripIII (Invitrogen. Carlsbad, California 92008, USA) in a 20µl reaction at 65 °C 5 min, 25 °C 5min, 55 °C 60min and 70 °C 15min.

2. Detection of FGF-2 and GAPDH expression level were performed with, 1µl of RT product and a 20 µl solution containing primers and probe from TaqMan Gene Expression Assays (GAPDH: S.O No: 185831521 / Plate ID: 647086 and FGF-2: S.O No: 185859498 /Plate ID: 647086) (Applied Biosystems, Foste City, CA, USA) were used.

3. 30µl of PCR reactions were set with TaqMan Universal PCR Master Mix (Part No: 4304437 / Lot No: MP2182) (Applied Biosystems, Foste City, Ca, USA) manufactured by Roche.

Total included: 15 µl of master mix, 1.5 µl of primer, 3.5 µl of water and 8 µl of cDNA per well.

4. Thermal condition: 50°C 2 min, 95 °C 10 min followed by 40 cycles of 95°C 15 sec and 60 °C 1 min. The experiment was performed in triplicates and analyzed by standard curve method.

I. STATISTICAL ANALYSIS

Statistics / t-test Analysis / Shallow Decay Group Vs Control.

Normality Test: Passed (P = 0.011)

Equal Variance Test: Passed (P = 0.942)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	15	0	186.153	171.260	41.537
DD	15	0	167.465	195.573	47.433

Difference 18.688

t = 0.296 with 32 degrees of freedom. (P = 0.769)

95 percent confidence interval for difference of means: -109.739 to 147.115

There is not a statistically significant difference between the input groups (P = 0.769).

Statistics / t-test Analysis / Deep Decay Group Vs Control

Normality Test: Passed (P = 0.011)

Equal Variance Test: Passed (P = 0.642)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	15	0	186.153	171.260	41.537
DD	15	0	167.465	195.573	47.433

Difference 18.688

t = 0.296 with 32 degrees of freedom. (P = 0.769)

95 percent confidence interval for difference of means: -109.739 to 147.115

There is not a statistically significant difference between the input groups (P = 0.769).

J. IRB Form

Health Sciences Institutional Review Board (IRB) • 540 East Liberty Street, Suite 202, Ann Arbor, MI 48104-2210 • phone (734) 936-0933 • fax (734) 998-9171 • irbhsbs@umich.edu

To: Tatiana Botero

From:
Charles Kowalski
Richard Redman

Cc:
Tatiana Botero
Jacques Nor

Subject: Notice of Determination of "Not Regulated" Status for [HUM00015518]

SUBMISSION INFORMATION:
Title: Caries-induced angiogenesis
Full Study Title (if applicable):
Study eResearch ID: HUM00015518
Date of this Notification from IRB: 8/29/2007
Date of IRB Not Regulated Determination : 8/29/2007

IRB NOT REGULATED STATUS:

Category	Description
Research Involving Coded Private Information or Biological Specimens	The IRB reviewed your application and determined that this study involves only coded private information or biological specimens that cannot be linked to a specific individual by the investigator(s) directly or indirectly through a coding system. In accordance with OHRP guidance on this subject (See http://www.hhs.gov/ohrp/humansubjects/guidance/cdebiol.htm), IRB approval is not required as the data cannot be tracked to a human subject.

Richard W Redman Charles J. Kowalski

Richard Redman
Co-chair, IRB Health Sciences

Charles Kowalski
Co-chair, IRB Health Sciences