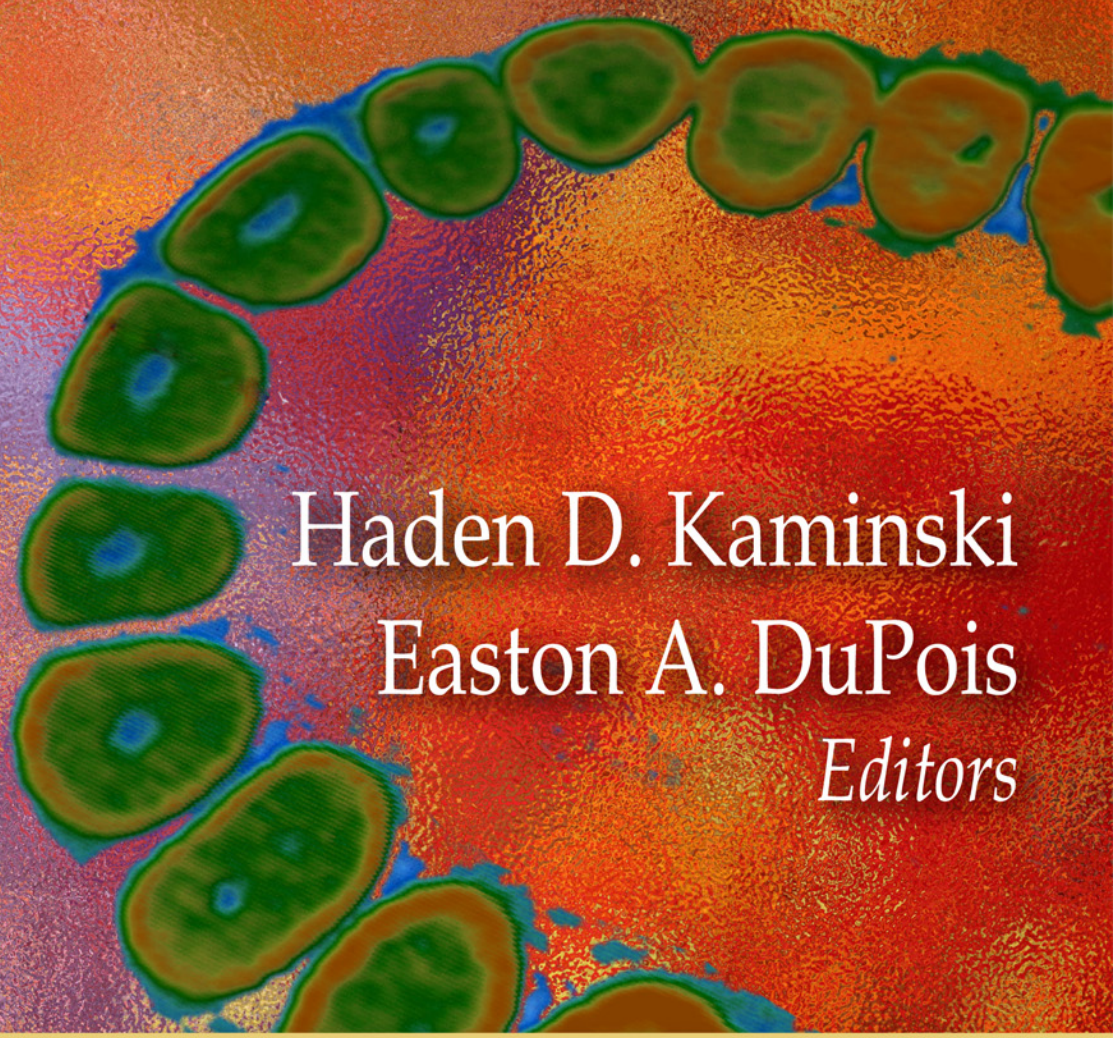
Dental Materials Research



Haden D. Kaminski
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Editors

Dental Science, Materials and Technology Series

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DENTAL MATERIALS RESEARCH

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Haden D. Kaminski and Easton A. DuPois (Editors)

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PREFACE

Dental restorative materials are specially fabricated materials, designed for use as dental restorations, which are used to restore tooth structure loss. This is usually resulting from, but is not limited to, dental caries. There are many challenges for the physical properties of the ideal dental restorative material, the properties of which can be divided into four categories: physical properties, biocompatibility, aesthetics and application. Chapters in this book include current research on today's materials and technologies in dental ceramics, biocompatibility of dental materials, and progress in bone and tissue regeneration. The need for simplified adhesive systems for the repair of small cavities in the primary teeth of children is also discussed in this book.

Chapter 1 - Ceramic materials are among the most biocompatible materials developed for dental restorations. The interest in all-ceramic restorations has rapidly increased over the past decade as stronger, tougher and more aesthetic materials are developed, along with novel processing technologies such as hot pressing and CAD/CAM (computer-aided design/ computer-assisted manufacture. Ceramics are widely used in dentistry because of their natural appearance and excellent mechanical, optical, thermal and chemical properties. Dental ceramics exhibit many desirable material properties, including biocompatibility, chemical inertness, aesthetics, compressive strength, diminished plaque accumulation, low thermal conductivity, abrasion resistance and colour stability. However, the possible applications for dental all-ceramic restorations are limited due to their brittle nature, sensitivity to flaws and defects, low tensile strength and fracture toughness. The presence of numerous surface and internal flaws, which may develop as a result of thermal, chemical or mechanical processes, can act as stress concentrators, reducing the strength of ceramics. These stresses can cause cracks to originate from the defect site which can propagate and lead to catastrophic failure. New materials and different processing methods have been introduced in recent years in an attempt to overcome these inherent problems. This review covers the development of dental ceramics including the history, classification and description of the different types of material, fabrication technologies, and mechanical and physical properties. Emphasis is placed on how these new materials and processing methods are overcoming the aforementioned problems. Clinical trials and longevity data is covered along with preparation guidelines. The evolution of all-ceramic restorations is addressed and focus is placed on high strength core ceramics and resin bonded glass-ceramic restorations. The interest in ceramic materials for dental applications has gradually increased due to the unique properties of ceramics and patient demand for optimal aesthetics as well as metal free alternatives.

Chapter 2 - Dental composite resins have revolutionized modern clinical dentistry. They are widely used for restoring teeth and cosmetic dentistry due to their esthetic and handling properties. Despite their wide applications, present day composite resins shrink when cured. This polymerization shrinkage generate stresses which affect the marginal seal between the tooth/restoration interfaces leading to secondary caries, post-operative sensitivity, tooth fracture, bond failure and marginal leakage. Other problems associated with current dental composites include inadequate wear resistance and the leaching of uncured organic monomers. The development of low shrinkage resins is therefore an important research focus in dentistry and remains a challenge. In this review, different polymerization techniques such as soft-start, pulse cure and pulse delay used to minimize shrinkage clinically will be discussed. The effect of the different light-curing techniques on the crosslink density of composites will also be reported. Recent developments of low shrinkage composites including some of our work on silsesquioxane in the laboratory will also be highlighted.

Chapter 3 - Confocal laser scanning microscope (CLSM) appears to be a valuable and non invasive tool for simultaneous 3D visualization of both cell morphology and structure and material scaffolds. The CLSM is also capable of creating excellent images using backscattered or reflected light. Confocal backscattered imaging is particularly useful for creating 3D images of the surface of materials and profilometric studies. For the quantification of maximal and minimal values in surface profile, distance between peaks in each surface, width of grooves and valleys, and axial ratio of the cells growing on the different surfaces were assessed. In addition to image data of cell morphology, microstructural data of scaffolds and cell-scaffold interaction can be readily obtained using this method. The quality of cell adhesion to the dental implant surface determines the tissue integration, and the surface can directly influence osteoblast adherence, attachment, spreading and metabolism, modifying and controlling the osseointegration process. The combinated use of reflectance and fluorescence modes is of capital interest to provide detailed images of living cell interactions with underlying surfaces in biomedical devices as dental implants, building the cellular and molecular basis of mechanotransduction, a process where high amounts of energy are demanded. Consistently with this, mitochondria that accumulate at sites of development of focal adhesions, mechanosensory devices, exhibit higher membrane potential compared with mitochondria that are not at those sites.

The methodology described herein is useful to directly visualize and quantify the role of underlying environmental cues for force-generating and anchoraging activities on mitochondrial bioenergetics in human living osteoblasts, growing on a number of customized biomaterials surfaces, and permit the use as a tool for designing the surface quality in order to provide the ideal surface for osseointegration of dental implants.

Chapter 4 - The advent of adhesive materials has allowed smaller preparations to be made, thus preserving sound tissues, in line with the Minimum Intervention (MI) philosophy. One needs to bear in mind that retentive preparations, with straight angles and convenient shape were necessary when the best restorative material available was amalgam, with no adhesive ability. Nevertheless, the option to restore and the type of material to use continue to be a matter of concern. Restorative dentistry undoubtedly plays a role in recovering function, aesthetics and allowing biofilm control by the patient/family, but healing the disease cannot be credited solely to the restoration, as it is associated with the control of etiological factors. Moreover, restorations in the oral environment are exposed to stresses of different origins that limit their longevity by interfacial degradation. Restoration failures lead to replacement that

implies further removal of dental structure, causing repetition of the restorative cycle. Thus, the decision to restore and the choice material involve the determining the patient's caries activity, immediate requirements and the notion of what the patient is able to receive in terms of dental procedures. In this sense, and considering the short biological course of primary dentition, materials once considered temporary, such as glass ionomer cement (GIC), can be used as definitive or even as an intermediate stage in the treatment plan. GIC presents chemical adhesion to the tooth structure, which is believed to be beneficial in terms of resistance to degradation, expansion coefficient similar to tooth structure, fluoride release and uptake, and GIC is especially indicated for temporary dentition. There is also the trend of incorporating GIC components into adhesive systems, with the intention of adding chemical adhesion to the conventional bonding mechanism—the glass ionomer adhesive system. Clinical time in the dental chair is also a problem where children are concerned, and it is important to develop techniques that speed up and facilitate the procedures. Therefore, the simplified adhesive systems play a role in these cases, and must be studied. Along the same lines, to reduce chair time, resin sealants can act therapeutically, when primary teeth have small cavities that reach dentin. In these cases, carious dental tissue is not removed; all tissue is preserved. Since it is known that "the seal is the deal", sealing the cavity will reduce bacterial counts and will probably stop the lesion.

Chapter 5 - Composite resins were introduced in the 1960s for the restoring of anterior teeth in substitution of the amalgam that presented both aesthetical and biocompatibility problems.

However, since the polymerization of methacrylates is never complete, it became evident the necessity to appraise the biocompatibility of composite resins. The incomplete conversion causes in fact the release of monomers that may implement adverse effects in the organism, i.e., allergic reactions, systemic toxicity, cytotoxicity, estrogenicity and mutagenicity. Because very little information has been so far delivered on the consequences of methacrylic monomers on cell metabolism, we were driven to investigate the biochemical interactions between methacrylates and human cells. The present work summarizes the effects of TEGDMA, UDMA, BDDMA, HEMA and Bis-GMA on 1) cellular energetic metabolism (oxygen consumption rate, glucose consumption, G6PDH, lactate production) and 2) cellular redox status (GSH concentration, and the activity of the enzymes regulating glutathione metabolism). The results obtained showed that all monomers induced both cellular differentiation and a decrease of oxygen consumption. Moreover, cells treated with TEGDMA and HEMA showed a significant enhancement of glucose consumption and lactate production, induced GSH depletion and stimulated G6PDH and GR activity. BDDMA also induced GSH depletion but without any effect on the activity of the enzymes involved in glutathione metabolism, while UDMA did not change GSH content and redox metabolism at all.

The mechanism of the differentiating action can be basically reconducted to an impairment of the mitochondrial respiration which starts two hours after incubation of the cells with each monomer and determines in the following hours an increase of anaerobic glycolysis. Thus the changes in energy metabolism and glutathione redox balance could be considered a potential mechanism for inducing clinical and sub-clinical adverse effects and thus providing a wider look for testing biocompatibility of dental materials.

Chapter 6 - This study compared the antibacterial effects of Clearfil Protect Bond (CPB), Clearfil SE Bond(CSB) and Xeno III(XIII) against cariogenic bacteria by disc diffusion method, agar well method and tooth cavity model using demineralized dentin.

For the agar well method, the test materials were filled in the inoculated agar wells. For the disc diffusion method, the paper discs were embedded in adhesives and placed on the agar plates. After 48 h, the inhibition zones were measured in mm. Four cavities were prepared on the dentin surface and demineralized by acid-gel method and left in bacterial suspensions for 3h. The materials were applied on the cavities and the fourth was left unapplied for control. Standart amounts of dentin chips were collected from the cavities and the number of bacterial recovery was counted.

CPB produced the significantly widest inhibition zones against all three bacteria within disc diffusion method and agar well method, where XIII couldn't produce any by disc diffusion method ($p<0.05$). For the tooth cavity model, CPB resulted in significantly less bacterial recovery than the other adhesives used on the demineralized dentin ($p<0.05$).

Within all the methods used CPB was found to have the most antibacterial effects against the three bacteria.

Chapter 7 - PRGF technology constitutes a breakthrough in the stimulation and acceleration of soft tissue healing and bone because it allows the local and continuous delivery of a wide range of growth factors and proteins, mimicking the needs of the physiological wound healing and reparative tissue processes. The versatility of this approach facilitates the combination of the different PRGF formulations with autologous bone and even with a wide range of biomaterials. Progress in the development of PRGF technology has stimulated its therapeutic use in numerous medical and scientific fields including dentistry, oral implantology, orthopedics, ulcer treatment, sports medicine and tissue engineering, among others.

Chapter 8 - **Statement of problem**: The fracture resistance of the root canal is mainly associated with the kind of post used for treatment, and studies indicate that there are still different opinions about the fracture resistance of root canals when non-metal posts are used.

Purpose: The purpose of this study was to compare the fracture resistance of four adhesive non-metal posts with different MOEs against compressive load in endodontically treated teeth.

Materials and Methods: In this in vitro experimental study, a total of 40 recently-extracted mandibular premolars were selected, sectioned adjacent to the CEJ, and endodontically treated. The specimens were randomly assigned to 4 groups (n=10). Post spaces were prepared and the fiber D T. Light posts, D.T. Composi post, D.T. White posts and Cosmopost were cemented. Composite resin (Lumiglass) cores were built up. After simulating the PDL the specimens were embedded in acrylic resin, and then secured in a Universal Testing Machine. A compressive load was applied at a 45° angle to the long axis of the tooth until fracture, at a crosshead speed of 1mm/min. Data were analyzed using (one-way) ANOVA and Tukey's test ($p<0.05$).

Results: Teeth restored with D. T. White fiber posts exhibited significantly higher resistance to fracture. Teeth restored with the other three groups, were statistically similar ($p>0.05$). Fractures observed in fiber groups would allow further restorations of the tooth, whereas unrestorable, catastrophic fractures were observed in Zirconia group.

Conclusion: Zirconia post, due to the unfavorable and unrestorable fractures caused, should be used with more consideration and therefore indication of fiber posts is suggested.

Short Commentary 1 - In vitro cytotoxicity studies have gained tremendously in popularity in recent years. To make these in vitro studies more clinically relevant, diffusion experiments in an in vitro pulp chamber device (IVPC) using dentin has repeatedly been advocated. The purpose of this study was to investigate dentin permeability of different thicknesses at an anatomical position of the tooth and then to determine the dentin thickness necessary to mask the cytotoxicity of a recent toxic dentin bonding agent (Xeno III).

Materials and Methods: Molar teeth were cut just coronal of the pulp horns creating discs of different thicknesses. These discs (99) were tested for their permeability in an In Vitro Pulp Chamber (IVPC) device. Furthermore, near confluent mouse 3T3 cells were exposed to Dulbecco modified eagle's medium with extractions from Xeno III through dentin discs of different thicknesses (35) as well as through standard membranes. The cell viability (survival rate) was measured using the MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and related to the non-exposed controls.

Results: When the relationship of disc thickness and permeability was studied, a substantial variation in the results at a specified thickness for different subjects was found but statistical analyses demonstrated a significant link between thickness and permeability. The decrease in dentin permeability became really evident at a thickness of more than ~350 μm.In the cytotoxicity experiments it was found that for the artificial membrane group as well as for etched dentin discs with a thickness of betrween 120–200 μm, cell viability was around 25% compared to the control which was 100%. When cytotoxicity was evaluated for the 500 μm disc as barrier, cell viability was at 60.7% with a cell death of 39%. In the 750μm group, cell viability was much higher at 75% but still cell death was noticed when compared to the control. Only at a disc thickness of 1000 μm and above no cell death was observed.

Conclusion: Dentin permeability cannot be predicted but should be determined separately for each disc as large variations between individual dentin discs were evident. Dentin permeability does not increase in a linear fashion with dentin thickness but rather exponentially. An alternative to dentin discs would be to choose a standard permeable membrane through which cytotoxicity can be measured. Furthermore, it cannot be assumed that no cytotoxicity will take place when using dentin barriers of 500 μm or even 750 μm. Xeno III should only be used in cavities with a remaining dentin thickness of at least 1000 μm between the cavity floor and the pulp.

Short Commentary 2 - Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application. Taking into account the tests available in general field, genotoxicity and cytotoxicity assays are of special concern since genetic damage and cellular death play important role in carcinogenesis. The purpose of this commentary is to provide an overview on the ability of compounds currently used in dentistry such as fluoride, chlorhexidine, endodontic compounds, dental bleaching agents, glass ionomer cements, fixed orthodontic appliances, dental implants and titanium miniplates in inducing genetic damage and/or cell death based on the authors' recent research. Some of these compounds appear capable of exerting noxious activity on the cellular machinery. The action mechanisms are discussed. Therefore, this is an area that warrants investigation since the estimation of risk of these substances with respect to genotoxicity and/or cytotoxicity certainly will improve oral health as well as will prevent oral carcinogenesis.

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Chapter 1

DENTAL CERAMICS: A REVIEW OF MODERN MATERIALS AND TECHNOLOGIES

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ABSTRACT

Ceramic materials are among the most biocompatible materials developed for dental restorations. The interest in all-ceramic restorations has rapidly increased over the past decade as stronger, tougher and more aesthetic materials are developed, along with novel processing technologies such as hot pressing and CAD/CAM (computer-aided design/computer-assisted manufacture [1]. Ceramics are widely used in dentistry because of their natural appearance and excellent mechanical, optical, thermal and chemical properties [2]. Dental ceramics exhibit many desirable material properties, including biocompatibility, chemical inertness, aesthetics, compressive strength, diminished plaque accumulation, low thermal conductivity, abrasion resistance and colour stability [2, 3]. However, the possible applications for dental all-ceramic restorations are limited due to their brittle nature, sensitivity to flaws and defects, low tensile strength and fracture toughness [4]. The presence of numerous surface and internal flaws, which may develop as a result of thermal, chemical or mechanical processes, can act as stress concentrators, reducing the strength of ceramics. These stresses can cause cracks to originate from the defect site which can propagate and lead to catastrophic failure [5]. New materials and different processing methods have been introduced in recent years in an attempt to overcome these inherent problems. This review covers the development of dental ceramics including the history, classification and description of the different types of material, fabrication technologies, and mechanical and physical properties. Emphasis is placed on how these new materials and processing methods are overcoming the aforementioned problems. Clinical trials and longevity data is covered along with preparation guidelines. The evolution of all-ceramic restorations is addressed and focus is placed on high strength core ceramics and resin bonded glass-ceramic restorations. The interest in ceramic materials for dental applications has gradually increased due to the unique properties of ceramics and patient demand for optimal aesthetics as well as metal free alternatives.

INTRODUCTION

Over the past decade, a number of new all-ceramic systems have been introduced for inlays, onlays, veneers, crowns and fixed partial dentures (FPDs) and for use in both the anterior and posterior region of the mouth. There has been an increased demand for these restorations from patients due to increased expectations regarding aesthetics and issues relating to the biocompatibility of metals and metal allergies [6]. An ideal all-ceramic system should possess the following characteristics: excellent mechanical and physical properties; biocompatibility; adequate marginal fit; optimum aesthetics; suitable for both anterior and posterior crowns and FPDs. Improvements have occurred with both the all-ceramic materials, with higher strength materials being introduced, and their processing methods, such as CAD/CAM (computer-aided design/computer-assisted manufacture) technology. With clinical performance and longevity of these all-ceramic restorations improving, they are now becoming more popular with predicable results. Ceramics are renowned for their natural appearance, chemical durability and excellent optical properties. However, they are brittle materials with low tensile strength and are susceptible to crack propagation. Various methods have been employed to overcome these inherent problems by increasing the resistance to brittle fracture by the development of reinforced ceramic core systems and resin-bonded ceramics. Many systems are now available on the market which can be confusing for the clinician. Each of these systems has unique properties and applications and for success it is imperative that the correct system is chosen for a particular clinical situation. Other considerations should include clinical preparation and correct cementation procedure.

This review covers the history of dental ceramics, different classification methods and a description of the different types of material and processing technologies. Emphasis is placed on how these new materials and fabrication methods are overcoming the aforementioned problems, and clinical aspects are also discussed. The evolution of all-ceramic restorations is addressed and focus is placed on high strength core ceramics and resin bonded glass-ceramic restorations.

BACKGROUND OF CERAMICS

The word ceramic is derived from the Greek word ‘Keramos’, which means ‘burnt stuff’, and actually pertains to ‘potter or pottery’ [7]. Until the 1940’s, the most important materials in this class were traditional ceramics such as china and porcelain, which are based on clay [8, 9]. Since then, there has been significant progress in developing the properties of ceramics and new materials have been fabricated for use in various industries such as aerospace, computer and electronic applications [8].

Due to their natural appearance and their excellent mechanical, optical, thermal and chemical properties, ceramics are widely used in dentistry [2]. They are nonmetallic, inorganic structures, which primarily contain compounds of oxygen with one or more metallic or semimetallic elements such as aluminium, calcium, lithium, magnesium, phosphorus, potassium, silicon, sodium, titanium and zirconium [8, 10, 11]. The properties of ceramics can be altered by the type and amount of the components used in their production. They may consist mainly of glasses, porcelains, glass-ceramics or highly crystalline

structures. Dental porcelains are primarily glass whereas dental porcelain that requires controlled thermal expansion contains a crystalline component. Other ceramics are composed entirely of crystalline oxides that are sintered together, sometimes under high pressure. Glass-ceramics are partially crystallised glasses that are produced by nucleation and growth of crystals in the glass matrix phase. These final particles and their distribution can increase the fracture toughness and strength of the material [12].

It is important to understand the structure of these materials, as they play a key role in the final properties of the material. Glasses are composed of silica (SiO_2) with other oxides e.g. CaO, Na_2O, K_2O, BaO and Al_2O_3. A typical soda lime silica glass composition is 70% wt SiO_2, with the remainder consisting of Na_2O (soda) and CaO (lime). Pure silica (SiO_2) in crystalline form is quartz mineral and glass formed from molten silica has a network structure. This random network structure of glass was first proposed by Zachariasen in 1932 [13]. Silicate glasses have a random structure based on an irregular arrangement of SiO_4 tetrahedra linked through corner sharing into a three-dimensional network. The structural basis of dental ceramics can be understood in terms of vitreous silica, SiO_2, which has been chemically modified to give suitable physical and chemical properties. The nature of these agents is generally in the form of oxides.

Three categories of oxide are involved in the structure of glass [13]. Network formers are oxides which form glasses when melted and cooled because of their ability to build up continuous three-dimensional random networks e.g. Si, B, and P. A second type is the network modifying oxides, which are incapable of building up a continuous network. Their function is to partly disrupt the network structure through the introduction of ionic bonds and the effect is usually to weaken the glass network. Various oxides are network modifiers including Na_2O, K_2O, CaO and BaO. They can reduce the viscosity of the glass and increase the coefficient of thermal expansion (CTE). For example, K_2O can increase the CTE through the formation of leucite crystals in the material. The metal ions involved tend to form non-directional ionic bonds with oxygen atoms, resulting in the formation of non-bridging oxygens in the structure. However, network modifiers are not always oxides and may be cations that are oxidised by fluorine. Intermediate oxides are a third type of oxide whose behaviour is in between that of a network former and a network modifier in that, although not capable of forming a glass network, they can take part in the glass network. Examples include Al_2O_3 and MgO, which act like glass formers when combined with others. Therefore, the composition of a glass can be controlled to give specific properties.

THE HISTORY OF DENTAL CERAMICS

Dental technology was first reported in Etruria in 700 BC and the Roman first century BC, but was not developed until the eighteenth century [2]. During this period, various materials were used for artificial teeth. The Etruscans made teeth of ivory and bone that were held in place by a gold framework [10]. Ivory, from hippopotamus or elephant, and animal teeth and bone carved to mimic human teeth, were used for many years thereafter. George Washington wore complete dentures that were carved from hippopotamus ivory by John Greenwood. However, ivory is porous and was found to soak up oral fluids. Eventually, they became badly stained as well as being highly unhygienic, and animal teeth proved to be

unstable in saliva [9]. Later, human teeth were used, which were sold by the poor, extracted from slaves or obtained from corpses [10].

In 1774, a Parisian apothecary at Saint Germain-en-Laye named Alexis Duchateau, with the assistance of a Parisian dentist Nicolas Dubois de Chémant, fabricated the first successful porcelain dentures at the Guerhard porcelain factory, replacing the stained ivory prostheses [2]. Dubois de Chémant improved and modified the porcelain paste composition and more usable results were obtained [14]. In 1789, he was awarded both French and British patents for an improved version of 'mineral paste teeth' [10]. Shortly after, Dubois de Chémant liaised with Josiah Wedgwood during the early years of this still famous porcelain manufacturer [2]. However, there were problems associated with these porcelain dentures and they were of limited success. The prostheses were manufactured from a single large piece, which hindered the denture's adjustment at the base of the mouth [14]. In addition, there were problems with uneven contraction of the various materials, probably due to shrinkage of the porcelain during firing, which led to a poor fit of the denture [15]. Over the years, other materials, such as vulcanite and polymethylmethacrylate, have superseded porcelain as a denture material.

In 1808, Giuseppangelo Fonzi, an Italian dentist, invented a porcelain tooth that was held in place by a platinum pin or frame [14]. These individually formed porcelain teeth were called 'terro-metallic incorruptibles' and demonstrated improved aesthetics and mechanical properties [2]. However, it was Pierre Fauchard who recognised the importance of porcelain as a means of reproducing colour and opacity of natural teeth in the key text 'Le Chirurgien Dentiste' (The Dental Surgeon).

Individual ceramic restorations were first recorded by Murphy in 1837, who used porcelain or rods of coloured glass fused onto a thin platina base to form an inlay. By the 1870s, many inlays were being ground from porcelain denture teeth to overcome problems with warpage of the foil and ceramic shrinkage during firing [15]. Later, Herbst in 1882, introduced glass inlays produced from a glass frit and then the Richmond crown was developed by Logan in 1885, which resolved the retention problem encountered between traditional porcelain crowns and wooden posts, by fusing the porcelain to a platinum post [2].

Dr Charles H. Land introduced the first fused feldspathic inlays in 1886 by using platinum foil directly and a high temperature gas furnace [2]. This technique provided restorations with both a clinically acceptable fit and aesthetic quality [15]. Land also introduced one of the first ceramic crowns, the porcelain jacket crown, in 1903, again using the platinum foil matrix and high fusing feldspathic porcelain. These crowns exhibited excellent aesthetics but the low flexural strength of the porcelain resulted in a high incidence of failures [10].

The first veneers were described back in 1928 when Dr Charles Pincus used porcelain veneers for Hollywood actors. These veneers were baked on platinum foil and retained on the teeth by denture powder [9]. Unfortunately, as the porcelain was thin and brittle, a high degree of fracture was encountered. With the introduction of acrylic resin in 1937, Pincus abandoned using porcelain for veneers in favour of this material.

Since then, feldspathic porcelains with reliable chemical bonding have been used in metal-ceramic restorations for nearly 40 years. Unfortunately, feldspathic porcelains proved to be too weak for all-ceramic crowns without a cast metal core or metal foil coping. In addition, their shrinkage during the firing process resulted in an inadequate fit and marginal adaptation [10]. In the 1950's, leucite additions to porcelain increased the coefficient of

thermal expansion (CTE) of the porcelain to enable their fusion to gold alloys substructures [2].

The first alumina-reinforced core, consisting of a glass matrix containing 40 to 50 wt% aluminium oxide, was introduced by McLean and Hughes in 1965 [16]. The core was baked on a platinum foil and subsequently veneered with matched expansion porcelain. The alumina particles improved the fracture resistance of the material, making it stronger, more effective at preventing crack propagation and acted as crack stoppers [9]. Unfortunately, because the core material was opaque and chalky white in appearance, veneering with feldspathic porcelain was necessary for aesthetic reasons. Even though the flexural strength was increased to 120-150MPa, compared to only 60MPa of feldspathic alone, it was only still indicated for the anterior region of the mouth [9].

During the last 35 years, there have been major developments in metal-ceramic systems, resulting in improved alloys, porcelains and porcelain-metal bonding [2]. A renewed interest in all-ceramic restorations occurred in the 1980's with the advent of a castable glass-ceramic crown system (Dicor) and a 'shrink-free' all-ceramic crown system (Cerestore) [2]. In 1983, Dr Horn proposed the use of hydrofluoric acid as an etchant for veneers constructed from a leucite-containing feldspar to enhance the bond between the ceramic and the resin based composite [17]. At a similar time, Calamia also reintroduced the method of etching porcelain for resin-bonded ceramics and suggested the use of phosphate-bonded refractory materials when fabricating all-ceramic veneers using conventional dental ceramic materials and techniques [32]. Since then, ceramics have become a fundamental material for dental restorations, especially where optimal aesthetics are required.

CLASSIFICATION OF MODERN DENTAL CERAMICS

Various ways of classifying dental ceramics have been suggested over the years according to their [10]:

- use or indications (anterior, posterior, crowns, veneers, post and cores, bridges, stain ceramic and glaze ceramic)
- composition (pure alumina, pure zirconia, silica glass, leucite-based glass-ceramic, lithia-based glass-ceramic)
- processing method (sintering, partial sintering and glass infiltration, CAD/CAM, copy milling, pressing)
- firing temperature (low-fusing, medium-fusing, high-fusing)
- microstructure (glass, crystalline, and crystal-containing glass)
- translucency (opaque, translucent, transparent)
- fracture resistance
- abrasiveness

An inherent problem with dental ceramics that has been apparent for many years has been a lack of strength and toughness. This may be overcome by supporting the dental porcelain with a stronger substructure and by manufacturing ceramics that are stronger and tougher.

Therefore, a sensible way to classify dental ceramics is into three categories based on the nature of the high strength supporting structure:

- Metal-ceramics
- Reinforced ceramic core systems
- Resin-bonded ceramics

METAL-CERAMICS

Metal-ceramic restorations have been reliably used since their development in the 1950's when Brecker described the baking of porcelain onto gold [18]. They were developed to overcome the problems of brittle fracture associated with all-ceramic crowns and, over the years, there have been improvements in both the alloy substructure and the veneering ceramic [19]. Leucite additions to the feldspathic porcelain increased the coefficient of thermal expansion (CTE), enabling their fusion to gold alloys substructures and, in 1962, Weinstein and Weinstein discovered that a chemical bond occurred between the porcelain and the metal [10]. These restorations have since gained widespread acceptance and are renowned for their excellent long term success rates. The metal provides the strength to the restoration whereas the ceramic creates the appropriate aesthetics. Good aesthetics are achievable but it is essential that an opaque ceramic layer is placed over the metal substructure to mask the metal. However, the appearance is still inferior to all-ceramic restorations. They frequently exhibit poor translucency and a metal collar or a grey shadow subgingivally. In addition, tooth preparation is destructive and in some patients, they are contraindicated due to a nickel allergy [6].

A metal-ceramic crown consists of a cast metal substructure (coping) on which several layers of an outer ceramic veneer are fired. A strong bond should exist between the ceramic and the metal, which is able to withstand the interfacial shear forces generated during fabrication due to the differences in CTE and to the shrinkage of the porcelain during firing [20]. Bonding of the ceramic to a metal substructure will eliminate microscopic cracks as the metal presents a barrier to the propagation of cracks due to its high fracture toughness [9]. Failure of metal-ceramic crowns is usually due to debonding of the ceramic from the metal. Therefore, the success of metal-ceramic restorations lies with the quality of this bond.

Veneering ceramics for metal-ceramic restoration contain a glassy matrix, 75-85% by volume, and various crystalline phases, usually leucite [21]. These crystalline phases are added to the veneering ceramic to increase the CTE to match that of the casting alloy and thus avoid tensile stresses developing within the veneer when cooled. If the mismatch is great, stress will build up during the cooling process following firing and will lead to crazing or cracking of the ceramic. The CTE of the ceramic should be slightly lower than that of the metal, which introduces residual compressive stresses within the veneering ceramic and provides additional strength for the restoration [9]. Multiple firing of these veneering ceramics can increase their leucite content and consequently will increase their CTE [22]. If the CTE increases above the value for the metal, the expansion mismatch between the porcelain and the core can produce stresses during cooling that are sufficient to cause

immediate or delayed crack formation in the porcelain. Therefore, it is advantageous to minimise the number of firings during manufacture of these restorations where possible.

There are a number of methods available for strengthening the inherently weak feldspathic veneer of these restorations. Most of these methods utilise the technique of placing the outer surface of the restoration under compressive stress to resist crack initiation and propagation [23, 24]. The ion exchange process creates high compressive stresses in the thin surface layer of the ceramic by smaller diameter sodium ions being replaced by larger diameter potassium ions [3, 25, 26]. This exchange has the potential to strengthen by subsurface compression. However, the compressive zone is less that 100μm deep and the strengthening effect could be lost if the ceramic surface is ground, worn, abraded or eroded [27]. Studies have shown an increase in flexural strength by more than 50% and fracture toughness by up to 100% in feldspathic porcelains following ion exchange [28, 29]. Glazing can also be used to strengthen ceramics by the formation of a low-expansion surface layer formed at high temperature which places the surface of the ceramic in compression and reduces the depth and width of surface flaws. However, glazing does not significantly improve the biaxial flexural strength of feldspathic porcelain [12, 30]. Thermal tempering is another method that produces a low expansion surface layer that causes compression during cooling, thus increasing resistance to crack initiation and growth. The presence of this surface compression has been shown to improve the biaxial flexural strength of body porcelain by as much as 158% [31].

The design of the metal substructure is of paramount importance because it provides the necessary strength and support for long term clinical success. The metal substructure should be designed to support porcelain thickness of no more than 2mm. In addition, the coping should ideally follow the anatomical form of the natural tooth to enable the support and usage of a thin veneering ceramic. Thick layers of veneering ceramic, especially in cuspal region, are unsupported and susceptible to fracture.

REINFORCED CERAMIC CORE SYSTEMS

Reinforced ceramic core systems can be subdivided into several categories: (1) Alumina reinforced feldspathic core; (2) Glass-infiltrated high strength ceramic core; (3) High density alumina core; (4) High density zirconia core.

ALUMINA REINFORCED FELDSPATHIC CORES

In 1965, the first alumina-reinforced core, containing 40-50 wt% alumina, was introduced by McLean and Hughes [16]. The alumina particles improved the properties of the material which was more effective at preventing crack propagation and acted as crack stoppers [9]. The flexural strength was 120-150MPa compared to only 60MPa of feldspar alone [9]. These crowns provided slightly improved aesthetics for anterior teeth in comparison to metal-ceramic crowns. However, they were only indicated for anterior crowns because of insufficient strength to withstand the occlusal forces in the posterior region of the oral cavity.

GLASS-INFILTRATED HIGH STRENGTH CERAMIC CORES

Most high strength ceramics derive their improved fracture resistance from a crack-blocking ability of the crystalline particles. The dispersion strengthening process involves reinforcing glasses and ceramics with a dispersed phase of a different material that is capable of hindering crack propagation through the material [10]. When a tough, crystalline material such as alumina is added to a glass, the glass is toughened and strengthened because the crack cannot pass through the alumina particles as easily as it can through the glass matrix [21]. Most dental ceramics that have a glassy matrix use reinforcement of the glass by a dispersed crystalline material.

The In-Ceram Alumina system (Vita Zahnfabrik, Bad Sackingen, Germany) was developed by Sadoun in 1984 and uses the addition of alumina to feldspathic glass to create high temperature sintered alumina glass-infiltrated copings [32]. A slurry of the material is slip-cast on a porous refractory die and heated in a furnace to produce a partially sintered coping. An alumina content of 85% is achieved by infiltration of the coping with a lanthanum glass, which penetrates the porous core to produce a dense ceramic. This processing method leads to a high strength material due to the presence of densely packed alumina particles and the reduction of porosity [21, 33, 34]. A conventional veneering ceramic is then applied to create the correct morphology and aesthetics. InCeram Alumina has a flexural strength of 236 to 600 MPa and fracture toughness of 2.7 to 4.61 $MPa.m^{0.5}$ [35-37]. Clinically, InCeram Alumina can be used to fabricate anterior and posterior crowns and, in addition, was the first restorative system introduced for the fabrication of 3-unit all-ceramic anterior bridges.

Two modifications of the In-Ceram technique have been introduced. With In-Ceram Spinel, a magnesium aluminate ($MgAl_2O_4$) spinel replaces the alumina as the major crystalline phase with traces of alumina. This improves the translucency of the final restoration because of the crystalline structure of the spinel and lower index of refraction compared with alumina [34]. In-Ceram Spinel, therefore, has superior aesthetics over InCeram Alumina. However, it is not as strong as the alumina-based material. The flexural strength is lower at 377 MPa and the clinical indications are for inlays only [38]. In-Ceram Zirconia combines the use of glass-infiltrated alumina with 35% partially stabilised tetragonal zirconia for the core material, combining the toughening mechanisms of zirconia with the partially sintered glass-infiltrated alumina [39]. It exhibits a flexural strength of 421 to 800 MPa and fracture toughness 6 to 8 $MPa.m^{0.5}$ [35, 36, 38, 40]. It has been successfully used for posterior three-unit fixed bridges [41, 42]. However, Heffernan *et al* [43] has demonstrated that in terms of translucency, In-Ceram Zirconia core is as opaque as a metal-alloy core and therefore is not recommended for anterior crowns and bridges where the translucency of the core material is a major factor in aesthetics.

All of the InCeram range can be produced by slip casting or by CAD/CAM processing using the CEREC or Celay systems. The CEREC system is discussed in detail later. The Celay system uses a copy-milling technique to manufacture ceramic inlays or onlays from resin analogues. A resin inlay or onlay is fabricated directly onto the prepared tooth or model, which is then removed, copied and reproduced in ceramic using the milling system [44, 45]. To produce blocks, powder is dry pressed by the manufacturer, which creates a denser, homogenous structure, which has a higher flexural strength after glass infiltration [46]. The

clinical preparation technique for these restorations involves a standard rounded shoulder margin and an axial reduction of 1.5mm.

PURE ALUMINA CORES

Pure alumina cores exhibit increased strength and superior translucency compared to the glass-infiltrated core materials. Procera AllCeram (Nobel Biocare, Stockholm, Sweden) was first described by Anderson and Ogen in 1993 and uses a densely sintered 99.5% pure alumina as the core material [47, 48]. With Procera AllCeram, a die is produce from the impression and the geometry of the coping is digitised using specialist software, which is transferred via a modem to a specialist dental laboratory in Stockholm. The coping ceramic is dry pressed onto the die and then sintered at 1600-1700^{0}C so it is fully densified with a negligible glassy phase [48]. Following this, the coping is returned and the veneering is undertaken with feldspathic porcelains. The 15-20% shrinkage of the core during sintering is compensated by fabricating an oversized ceramic pattern that will shrink during sintering to the desired size for accurate fit. Flexural strength is reported to be 487-699MPa and the fracture toughness 4.48 to 6 MPa.m$^{0.5}$ [35, 36]. Clinical indications include anterior and posterior crowns, veneers, onlays, inlays, short span bridges and implant abutments. A conventional preparation technique is used with a moderate chamfer margin and rounded internal line angles. Preparation burs specific for Procera are available.

Another example is TechCeram from TechCeram Ltd, UK. In this case, a special die is produced from the impression at the TechCeram Ltd laboratory and a fine particle pure alumina is sprayed onto a refractory die through an oxygen/acetylene flame. As they pass through the flame, the alumina particles melt and have sufficient momentum to 'splat form' onto the refractory die model, rapidly producing a core 0.3-0.4mm thick [49]. This technique, where each particle contracts before the next lands on top of it, overcomes the problem of sintering shrinkage [49]. This alumina core has 80-90% density and is sintered at 1170^{0}C. As before, the coping is returned to laboratory and veneered with a conventional feldspathic ceramic. The biaxial flexural strength of this material is reported to be 300MPa and can be used to fabricate simple anterior and posterior crowns and inlays [47]. Clinical preparation involves a reduction of 1.5-2mm incisally/occlusally and a rounded shoulder of 1.5mm.

HIGH DENSITY ZIRCONIA CORES

The most recent core materials for all-ceramic restorations are the yttrium tetragonal zirconia polycrystals (Y-TZP) based materials. Originally they were introduced for biomedical use in orthopaedics for total hip replacements and were found to exhibit excellent mechanical properties and biocompatibility. Then, in the early 1990's, Y-TZP was used for endodontic posts and implant abutments. Nowadays, it is being used a core material for all-ceramic crowns and bridges, both anteriorly and posteriorly [50]. The addition of a metal oxide such as yttrium oxide partly stabilises the pure zirconia at room temperature in the tetragonal phase, resulting in the formation of a multiphase Y-TZP ceramic. The ability of Y-TZP to transform from tetragonal to monoclinic structure, which helps to prevent crack

propagation, is a key factor of the strength and toughness of the ceramic [50]. The mechanical properties of zirconia are mainly attributed to this transformation toughening, which can be induced by external stresses such as grinding, cooling and impact [1]. This is associated with a local increase of 3% to 5% in volume, which causes compressive stresses that may develop on the surface or around a crack tip and can close the crack tip, preventing further crack propagation [51]. In addition, zirconia ceramics are further toughened by the process of 'crack shielding' involving the formation of a micro-cracked zone around a propagating crack, which reduces the crack-tip stress and generates compressive stresses that prevent further propagation [52]. The flexural strength of these ceramics ranges from 900–1200 MPa and fracture toughness from 9–10 $MPa.m^{1/2}$ [50, 52, 53]. Regarding clinical preparation, the technique consists of 1.5-2.0mm incisal/occlusal reduction, 1.0-2.0mm axial reduction with rounded internal line angles and a chamfer margin.

There are two CAD/CAM methods of manufacture of Y-TZP restorations. They can be milled out of homogenous ceramic green-body blanks of zirconia which are subsequently sintered and shrunk to the desired final dimensions (e.g. Cercon, DeguDent; Lava, 3M ESPE) or the restorations can be machined directly with the final dimensions out of highly dense sintered prefabricated zirconia blanks (e.g. DC-Zirkon, CS Dental, Switzerland) [47, 54]. Following this, the substructures are veneered with an appropriate veneering ceramic as recommended by the manufacturer.

DISADVANTAGES OF REINFORCED CERAMIC CORE SYSTEMS

There are disadvantages to all of these high strength reinforced ceramic core systems. Increasing the mechanical strength of the cores by increasing the crystalline content and decreasing the glass content has resulted in acid-resistant ceramics whereby any type of acid treatment produces insufficient surface roughness necessary for adequate bonding to resin [55-57]. However, some bonding will occur due to the surface roughness of the coping produced from the processing methods. In addition, they are difficult to bond chemically to resins via silane coupling agents because of their reduced silica-based content [48, 56]. In general, ceramics containing less than 15 wt% silica are not regarded as silica-based or silicate ceramics [58]. Silica coating the ceramic surface seems to be a promising method to promote the bonding of acid resistant ceramics to resin [39]. It has been suggested that this surface treatment procedure can increase the silica content on ceramics and metals enhancing the bond to resin via silane coupling agents [48, 59].

There have also been reports of chipping of the veneering ceramic with zirconia frameworks, reasons being considered to be due to a mismatch in the coefficient of thermal expansion between the two materials and type of surface finish of the framework[60, 61].

A summary of the flexural strength and fracture toughness of a selection of commercial core reinforced dental ceramics is presented in Table 1.

Table 1. Flexural strength and fracture toughness of core reinforced ceramics ([a] Wagner et al. 1996, [b] Yilmaz et al. 2007, [c] Seghi et al. 1995, [d] Rizhalla et al., [e] Raigrodski et al. 2004 , [f] Vita Zahnfabrik et al. 2005, [g] Chai et al. 2007, [h] Guazzato et al. 2004, [i] Rimmer et al. 2006, [j] Pittayachawan et al. 2007)

System	Core material	Biaxial flexural strength (MPa)	3 point flexural strength (MPa)	Indentation fracture toughness ($MPa.m^{1/2}$)
InCeram Alumina	Glass-infiltrated alumina	352[a] 342[b]	446[c] 548[d]	3.1–4.6[e] 4.8[b]
InCeram Spinel	Glass-infiltrated with magnesium spinel		378[c]	2.4[f]
InCeram Zirconia	Glass-infiltrated with 35% partially stabilised zirconia	542[b] 927[g]	604[c] 476–630[h]	5.6[b] 6–8[e] 4.9–4.8[h]
Procera AllCeram	Densely sintered high purity alumina	687[a]		4.5[a] 4.48–6[e]
TechCeram	High density flame sprayed alumina	300[i]		N/A
Lava	Y-TZP	1100[j]	900-1200[e]	9–10[e]
Cercon	Y-TZP	1141[b] 927[g]	900-1200[e]	6.3[b] 9–10[e]
DC-Zirkon	Y-TZP		900-1200[e] 840[h]	9–10[e] 7.4[h]

RESIN BONDED CERAMICS

Resin bonded crowns have been in use for over 15 years and are defined as 'a full coverage restoration in which an all-ceramic crown is bonded to the underlying dentine (and any available enamel) using a resin bonded luting material with the bond being mediated by the use of a dentine bonding system and a micromechanically retentive ceramic surface' [62]. With these restorations, the tooth acts as the supporting substrate and the resin bonding procedure improves the fracture resistance of the entire system. Consequently, this enables the tooth preparation to be minimal and is therefore, a much conservative approach compared to the metal-ceramic and reinforced ceramic core systems.

In recent years, there has been a major development of new ceramics for use as resin bonded all-ceramic restorations due to the ability to bond composite resin to enamel, dentine and ceramic. New ceramic systems have been introduced along with strong, reliable adhesion provided by new bonding luting systems [63]. This has allowed the extension of the clinical applications of these all-ceramic restorations from veneers to anterior and posterior crowns and inlays.

A reliable bond between the resin cement and ceramic is essential for clinical success with resin bonded all-ceramic restorations. Physical bonding is achieved by micromechanical interlocking of the resin and ceramic surface, and may be enhanced by surface roughening of the ceramic by hydrofluoric acid etching or gritblasting with aluminium oxide [64]. Acid-sensitive glass-ceramics (feldspathic, leucite and lithium disilicate) undergo surface degradation by hydrofluoric acid, creating a surface texture that favours micromechanical

bonding. Chemical bonding may be promoted by using silane coatings (bifunctional coupling agents) that aid covalent bonding between the organic resin and the inorganic ceramic [65-67]. In addition, silane coupling agents bond the silica oxides present in ceramics to the organic matrix of resin cements by means of siloxane bonds [68].

It has been shown that by resin bonding a weaker but etchable crown to the underlying dentine, the restoration will exhibit superior fracture resistance to that of conventional alumina or metal reinforced crowns [69, 70]. In addition, the adhesive bond is found to eliminate surface flaws which consequently decrease the potential for fracture [9]. Marquis [71] suggested that the resin cement modified the surface flaw population by crack healing, which increased the resistance to fracture. In contrast, Nathanson [72] proposed that the polymerisation shrinkage of resin cements 'stresses' the molecules together, which strengthens the ceramic. Fleming *et al* [69] investigated these two theories and found that resin cements significantly increased the strength independent of defect population. It was noted that the combination of surface pre-treatment and resin cementation moved the fracture origin from the ceramic/cement interface to the cement surface, which is consistent with improved resin strength independent of defect severity [69].

GLASS-CERAMICS

Glass-ceramics are polycrystalline solids formed through the controlled crystallisation of glass [13]. Glasses are melted, fabricated to shape, and then converted by heat treatment to a predominantly crystalline ceramic. The basis of controlled crystallisation lies in efficient internal nucleation, which allows development of fine, randomly orientated grains without voids, microcracks, or other porosity [73]. There are two parts to the crystallisation process: nucleation and crystallisation. Nucleation may be homogenous, when the first nuclei are of the same constitution as the crystals which grow on them; or heterogeneous, where the nuclei are different from the crystals which are deposited. Heat treatment or ceramming usually involves two or more stages; a lower temperature step to induce nucleation, allowing the formation of a high density of nuclei (nucleation hold) and one or more higher temperature treatments to promote crystal growth (crystallisation hold) [74]. The length of time the material is held at the crystallisation temperature depends on the degree of crystallinity that is required in the final ceramic [13].

The properties of glass-ceramics depend upon both the composition and microstructure. The bulk chemical composition controls the ability to form a glass and its degree of workability [13]. In order to achieve internal nucleation, suitable nucleating agents are melted into the glass, which promotes volume nucleation and the production of a glass-ceramic. Metallic oxides, such as zirconia and titanium dioxide are commonly used in silicate systems. Bulk composition also directly determines the potential crystalline phase structure, which governs the physical and chemical characteristics such as hardness and acid resistance [13]. Microstructure is also of paramount importance and is a key factor in most mechanical and optical properties. It is therefore necessary to characterise glass-ceramics in terms of both composition and microstructure. The mechanical properties of glass-ceramics such as improved strength and fracture toughness and their ability to be machined are closely related to the nature of the crystalline phase that develops during crystallisation heat treatment [13].

The major advantages of glass-ceramics over conventional ceramics are that they have fully densified bodies and complex shapes can be manufactured using standard hot glass techniques [75]. They also have a variety of unique physical properties such as strength, machinability, transparency and thermal shock resistance [75]. Therefore, glass-ceramics have potential applications in dentistry due to their chemical inertness, high mechanical strength and appropriate thermal and physical properties [76].

GLASS-CERAMICS FOR DENTAL RESTORATIONS

The first glass-ceramic was introduced to dentistry by MacCulloch in 1968 and was used in the production of posterior denture teeth [15]. In the 1970's, McLean and O'Brian described the use of leucite-based sintering ceramics for veneering metal frameworks and subsequently research was focussed at replacing the metal substructures in dental restorations [9]. The introduction of the ceramic acid etching technique in the early 1980's allowed the first adhesive ceramic veneers to be made on anterior teeth [17].

It was not until 1984 that the first dental glass-ceramic, Dicor, was launched. It was developed from a formulation of low thermal expansion ceramic used for cookware by Corning Glass Works and marketed by Dentsply International. Dicor was a micaceous glass-ceramic (45 vol% glass and 55 vol% crystalline tetrasilicic fluormica), processed by a combination of conventional lost wax investment techniques and glass casting [2]. The clinical applications for this glass-ceramic were veneers, inlays and crowns in the anterior region only, because its properties proved to be insufficient for posterior restorations.

This material was originally supplied as glass ingots containing a nucleating agent (MgF_2) that were melted and cast into a refractory mould and subsequently heat treated to allow the growth of the tetrasilicic crystals within the material, which provided the final improved properties [76]. The restoration was then coloured with a thin outer layer of shading porcelain and surface stain to achieve acceptable aesthetics. The crystals were responsible for providing the material with strength, resistance to crack propagation, good colour stability and optical properties, abrasion resistance, thermal shock resistance and excellent biocompatibility [76]. Table 2 summarises the advantages and disadvantages of this first commercial dental glass-ceramic, Dicor.

Further development of this material resulted in the introduction of Dicor MGC, a machinable glass-ceramic. This was a higher quality product, containing 70 vol% tetrasilicic fluormica, which was crystallised by the manufacturer and provided as CAD/CAM blanks or ingot. The mechanical properties of Dicor MGC were similar to Dicor glass-ceramic although it exhibited reduced translucency [10].

Dicor has since been discontinued due to low tensile strength and the need to colour the restorations on the exterior region rather than within the core region [77]. The survival rate of Dicor in high stress areas was poor in comparison to metal-ceramic restorations [10]. In addition, the fit of Dicor restorations was inferior to metal-ceramic restorations, though still below the 100μm limit. However, another study has reported a survival rate of 80% for Dicor restorations over a 14 year period [94].

Currently, glass-ceramics are used as biomaterials in two different fields: (1) as highly durable materials in restorative dentistry; and (2) as bioactive materials for the replacement of

hard tissue [78]. For use as a restorative material, glass-ceramics have to demonstrate durability in the oral environment, have excellent aesthetics and exhibit high strength and wear resistance.

Table 2. Summary of the advantages and disadvantages of the first commercial dental glass-ceramic, Dicor

Dicor Glass-ceramic	
Advantages	**Disadvantages**
Ease of fabrication Increased aesthetics Minimal processing shrinkage Good marginal fit Moderately high flexural strength Low thermal expansion similar to enamel Minimal abrasiveness to enamel	Limited use in high stress areas Inability to colour internally

COMMERCIAL GLASS-CERAMIC RESTORATIVE MATERIALS

Vitabloc Mark II (Vita Zahnfabrik) blocks are a second generation of fine particle feldspar ceramic blocks which contain sanidine as the crystalline phase. Available since 1991, they have replaced the original Vitabloc Mark I ceramic block. They are fabricated using fine grained powders which creates a nearly pore free ceramic with a fine crystal structure. This microstructure is responsible for increased polishability, decreased enamel wear and increased strength [79]. The flexural strength is approximately 130MPa when polished and when glazed, increases to 160MPa, which is twice that of conventional feldspar porcelains [38, 79]. As the ceramic can be acid etched, the system is amenable to adhesive bonding. This material is indicated for the fabrication of veneers, inlays and onlays and single crowns in both the anterior and posterior region [80]. These blocks can be machined with both the CEREC 3 and inLab systems.

A disadvantage of Vitabloc Mark II is that they are monochromatic although they can be stained and glazed. This is a common problem with many block materials for CAD/CAM processing. For improved aesthetics, two other variations of the Vitabloc Mark II are available. The Vitablocs Esthetic Line is an even more translucent version of the Mark II ceramic with increased glass phase content, which makes it particularly suitable for anterior crowns and veneers. The Vitabloc TriLuxe (Vita Zahnfabrik) contains a graded variation in colour saturation consisting of three shades. The middle layer has a regular chroma the top layer (enamel) has a low, less intense chroma with high translucency and the lower layer (neck) has the highest chroma and low translucency [79]. Using these blocks, it should be possible to achieve a more natural and aesthetic result.

ProCAD (professional computer assisted design), manufactured by Ivoclar Vivadent, is a CAD/CAM leucite reinforced glass-ceramic and has a flexural strength of 135-160 MPa and a fracture toughness of 1.3 MPa. $m^{1/2}$ [81]. After machining the surface of the glass-ceramic can be improved by polishing or applying a specially developed ProCAD glaze. This improves the flexural strength to 180-240 MPa [81]. It is possible to etch the glass-ceramic by applying

hydrofluoric acid and to form a retentive layer for adhesive cementation. ProCAD is available in a number of different shades and translucencies and characterisation can be achieved with external stains. Clinical indications include anterior and posterior crowns, inlays and onlays. It demonstrates good stability, good aesthetics and accurate machinability due to the small uniform size of the crystals [82]. A disadvantage is that the crowns need to be glazed to achieve adequate stability. Again, these blocks can be machined using the CEREC 3 and inLab system.

IPS Empress (Ivoclar Vivadent) was released in 1990 and utilises custom-made leucite containing ceramic ingots for the hot pressing technique [83]. This leucite reinforced glass-ceramic obtains its strength by finely dispersed leucite crystal reinforcement and was designed for restoring single units including veneers, inlays, onlays and crowns [84]. The ingots contain leucite crystals which are obtained by surface nucleation [85]. The hot pressing step disperses the leucite crystals further and the final microstructure exhibits 40% by volume of 1-5μm tetragonal leucite in a glassy matrix [21]. The final restorations may be completed by the application of stains and glazes, or alternatively trimmed back and veneered with thermally compatible ceramics prior to glazing [86]. The restorations are subsequently bonded to the tooth structure with a luting material, preferably an adhesive bonding system. The flexural strength of Empress significantly improves after additional firings [87]. The strength increase is attributed to a good dispersion of the fine leucite crystals as well as the compressive stresses arising from the thermal contraction mismatch between the leucite crystals and the glassy matrix [21].

However, there has been a decline in usage of Empress as it was found not to offer the improved strength that the manufacturers claimed and is prone to fracture in posterior regions [88]. In addition, problems have been encountered concerning interactions between the investment and the material, leading to the product being withdrawn from the USA.

Recently, *Empress Esthetic* and *Empress CAD* have become available for hot pressing and CAD/CAM technology respectively. Both materials are leucite reinforced glass-ceramics with flexural strength of 160MPa and fracture toughness of 1.6-1.8 $MPa.m^{1/2}$ [89]. Excellent aesthetics are achieved due to the materials high translucency. Clinical indications include inlays, onlays, crowns and veneers.

IPS Empress 2 (Ivoclar Vivadent) was developed to enable the fabrication of crowns and three-unit fixed bridges up to the second premolar. This system uses a lithium-disilicate glass core material and the framework is fabricated by the lost wax and hot pressing technique. Its chemical composition is primarily 60 wt% lithium disilicate, which represents the main crystalline content [84]. This material offers high strength and fracture toughness and, at the same time, a high degree of translucency [78]. With a flexural strength of 300-400MPa and a fracture toughness of 2.8-3.5 MPa $m^{1/2}$, this is almost three times those of leucite glass-ceramic [35]. To further improve the aesthetic and wear properties, it is veneered with an apatite-containing glass-ceramic using a sintering process. The more translucent Empress 2 core material eliminates the use of opaque alumina or metal substructure and potentially aids better aesthetics [86]. It is recommended that lithium disilicate restorations are adhesively bonded to etched tooth structure to increase their strength and longevity.

IPS e.max was introduced in 2005 to meet the growing demand for CAD/CAM applications. There are five components to the IPS e.max system:

- IPS e.max Press (lithium disilicate glass-ceramic ingot for the hot pressing technique)
- IPS e.max ZirPress (fluorapatite glass-ceramic ingot for the press-on veneer technique)
- IPS e.max CAD (lithium disilicate glass-ceramic block for the CAD/CAM technique)
- IPS e.max ZirCAD (zirconium oxide block for the CAD/CAM technique)
- IPS e.max Ceram (fluorapatite veneering ceramic)

The lithium disilicate glass-ceramic for CAD/CAM processing is IPS e.max CAD. Manufacture of the blocks is by a pressure casting procedure which uses optimised processing parameters that prevent the formation of defects, such as pores and accumulation of pigments, in the bulk of the ingot [90]. The blocks are supplied in their partial crystallised state to allow fast CAD/CAM machining. This intermediate phase consists of 40% lithium metasilicate crystals (Li_2SiO_3) of 0.2 to 1.0μm embedded in a glassy phase, which are responsible for the material's good machining properties, relatively high strength and good edge stability [90]. After the milling process, the restorations are heat treated at 850^0C to form 70 vol% fine grain lithium disilicate ($Li_2Si_2O_5$) embedded in a glassy matrix. This is the fully crystallised state, which imparts the high strength to the material. Colouring ions are included to achieve the desired aesthetics of the glass-ceramic. In the partially crystallised state, the blocks exhibit a blue colour due to a different oxidation state of the polyvalent colouring elements [90]. The final colour and opacity is achieved by heat treatment and subsequent cooling phase. The substructure is veneered with a fluorapatite ceramic, followed by characterisation and glazing. The apatite crystal phase acts as a component that adjusts the optical properties of the restoration to natural tooth [78]. Again, cementation is achieved with a composite resin luting system. E.max CAD can be used to fabricate anterior and premolar crowns, implant superstructures for single teeth restorations (anterior and premolar) and for primary telescope crowns.

Table 3. Summary of the biaxial flexural strength and fracture toughness of some commercially available glass-ceramics for dental restorations. ([a] Thompson et al 1996, [b] Buhler-Zemp et al. 2005, [c] Luthy et al. 1996, [d] Seghi et al., [e] Gaglio et al. 2001, [f] Dong et al. 1992, [g] Albakry et al. 2003, [h] Guazzato et al. 2004, [i] Holand et al. 2000, [j] Lawn et al. 2004, [k] Chai et al. 2007)

System	Reinforcing component	Biaxial flexural strength (MPa)	3-point flexural strength (MPa)	Indentation fracture toughness (MPa $m^{1/2}$)
Vitabloc Mark II	Sanidine	130–160[a] 110[b]	120[c] 122[d]	1.26[a]
ProCAD	Leucite	135–160[e]	140-200[b]	1.3[e]
Empress	Leucite	134[f] 175[g]	106[h] 112[i]	1.4[j] 1.2[h] 1.3[i]
Empress 2	Lithium disilicate	359[k] 407[g]	306[h] 400[i]	2.9[g] 3.3i
E max CAD	Lithium disilicate	360[b]		2–2.5[b]
Empress Esthetic	Leucite	160[b]		1.6–1.8[b]
Empress CAD	Leucite	160 [b]		1.6–1.8[b]

Table 3 lists the biaxial flexural strength and fracture toughness and Table 2.4 summarises the main crystal phase, mechanism of nucleation and crystallisation, processing methods and application of various commercial glass-ceramics for dental restorations

Because glass-ceramics can be resin bonded to the underlying tooth structure, clinical preparation can be more conservative in comparison to the reinforced ceramic ore systems. A typical preparation would entail an axial reduction of approximately 1.0mm and an incisal/occlusal reduction of 1.5-2.0mm.

PROCESSING METHODS OF COMMERCIAL DENTAL CERAMICS

A variety of different processing methods have been used over the years to fabricate ceramic restorations. These include sintering, slip-casting, hot pressing and CAD/CAM manufacture.

The *sintering* process is defined as the transformation of an originally porous compact to a strong, dense ceramic [91]. Sintering occurs at a temperature above the softening point of the porcelain where the glassy matrix partially melts and the powder particles coalesce [25]. A slurry of the ceramic powder is applied to a refractory die or platinum foil, dried and subsequently fired in a porcelain furnace. The density of the porcelain greatly increases during this process and there is a volume shrinkage of 30-40%. Porosity can be reduced from 5.6 to 0.56% by vacuum firing [92].

Using multiple layering of the ceramic, the correct morphology, colour and translucency of the restoration can be created [93]. An example of a sintered leucite reinforced glass-ceramic is Fortress (Mirage, Kansas, USA). The disadvantages of all-ceramic restorations made by the sintering process are that sintering the particles together can result in microporosities and inhomogeneties between the particles, which can initiate crack formation [4].

The *slip casting* technique involves the condensation of aqueous porcelain slip on a refractory die. The porosity of the refractory die aids condensation by absorbing water from the slip by capillary action[21]. After firing at high temperature on the die, the refractory shrinks more than the condensed slip. The fired porous core is subsequently glass infiltrated, a process in which molten lanthanum-containing glass is drawn into the pores by capillary action at high temperature. The advantages of slip casting include reduced porosity, fewer processing defects and higher fracture toughness.

The *hot pressing* of a ceramic material to form a dental restoration was developed by Ivoclar Vivadent (Liechtenstein) and was first described by Wohlwens and Scharer in 1990 [83]. The processing method utilises the lost-wax technique where an accurate wax pattern of the restoration is produced and then invested in a refractory die material [21, 78, 86]. The wax is then burnt out to create the space to be filled by the glass-ceramic. As the glass-ceramic comprises a certain volume of glass phase, the material can be pressed into a mould using the principle of viscous flow [78]. The ceramic ingots are pressed via an alumina plugger at 1150^0C under a pressure of 0.3- 0.4MPa into the refractory mould created by the lost wax technique and held for 20 minutes [94]. The process of hot pressing leads to a better crystal distribution within the glass matrix which improves the strength of the material [95]. In addition, hot pressing helps avoid large pores caused by non uniform mixing and prevents

extensive grain growth or secondary crystallisation [21]. Pressable glass ceramics are popular due to their ease of fabrication, occlusal accuracy and excellent marginal fit, translucency, and good mechanical properties, including increased flexural strength and decreased porosity [84, 94, 96].

The application of *computer-aided design/computer-assisted manufacture* (CAD/CAM) technology in dentistry represents a major breakthrough for both dental laboratories and surgeries [97]. The concept of using CAD/CAM techniques for the fabrication of dental restorations originated with Duret in the 1970's. Then, in 1980, Mormann and Brandestini commenced the development of a CAD/CAM method for use in dental surgeries at the University of Zurich [98]. The first chairside fabrication and placement of an inlay using this technology was undertaken in 1985 and the following year, Siemens (now Sirona) acquired the licence to market and further develop the system now known as CEREC. CEREC stands for **C**eramic **Rec**onstruction. Continual development of the hardware and software has resulted in rapid improvement in the CEREC system. The CEREC 3 was introduced in 2000 and can fabricate inlays, onlays, crowns and veneers. The new three-dimensional software allows for much easier handling, interpretation and manipulation when designing the restorations [99].

The system consists of a miniature video camera, which is used to take pictures of the prepared cavity, then relayed to the computer and a three-dimensional image is produced. The restoration is then designed using the software programme and the restoration is machined from a ceramic block placed in the milling unit of the CEREC 3 system. It is the only chairside CAD/CAM system available at present [100].

The CEREC system offers the opportunity to prepare, design and fabricate a ceramic restoration in a single appointment, without the need for making impressions, temporaries or dental laboratory support [98]. These systems mill restorations from industrial ceramic blocks which are prefabricated under optimum and controlled conditions [101]. Such blocks demonstrate a high and uniform ceramic quality, without the defects associated with manually produced restorations [102]. However, the disadvantages associated with CAD/CAM systems include high cost of the equipment, technique sensitivity in both scanning and designing the restorations, and hence a steep learning curve.

A number of laboratory based CAD/CAM systems have become available in recent years as a result of the greatly improved performance of computer hardware and software. The CEREC inLab, first introduced in 2002, is designed to produce multiple-unit restorations using high strength ceramic frameworks for crowns and bridges as well as restorations such as inlays and crowns.

With the inLab system, a stone model is poured from the impression using laser-visible stone and then automatically scanned with the laser scanner of the inLab or by using the separate inEos scanner. A three-dimensional image of the model is produced and using the software programme, the design of the substructure or restoration can be created. The software allows customisation such as anatomically shaped frameworks and occlusal surface design based on biogeneric tooth models [103]. After completion of the design, the selected material block is inserted into the milling chamber and automatically machined. In the case of frameworks, any necessary sintering is undertaken before an appropriate veneering ceramic is applied.

InEos is a new scanning system used in conjunction with the inLab system, which allows independent scanning and milling. The previous system was a combined unit, therefore only

scanning or milling could be undertaken at one time. Two laser scanning modes are available: (1) rotational, which is indicated for single units and records eight photos while the die is rotated; (2) overview, indicated for bridges, crowns, inlays and onlays. It can also be used to scan an opposing model for creating the occlusion of a restoration.

SURVIVAL RATES AND MODE OF FAILURE

Typical survival rates for all-ceramic restorations have been reported to range from 88 to 100% after 2–5 years and 84 to 97% after 5–14 years the most common method of failure is fracture/delamination of the veneering ceramic and/or fracture of the ceramic coping [104]. Other modes of failure that have been observed include root or tooth fracture, caries and endodontic therapy [105]. Table 4 details the survival rates from a number of clinical trials of all-ceramic restorations.

Table 4. Clinical trial data including survival rates for all-ceramic restorations

All-ceramic system	Type of restoration	Number of restorations	Evaluation period	Survival rate %	Investigator
In-Ceram Alumina	FPD	42	5	93	Olsson et al. 2003
In-Ceram Spinel	Crowns	40	5	97.5	Fradeani et al. 2002
In-Ceram Alumina	Crowns	24	3.9 ± 0.9	92	Bindel et al. 2002
In-Ceram Spinel	Crowns	19	3.9 ± 0.9	100	Bindel et al. 2002
In-Ceram Zirconia	FPD	18	3	94.4	Suarez et al. 2004
Procera AllCeram	Crowns	205	5	96.7	Fradeani et al. 2005
Lava	FDP	35	3	100	Pospiech et al. 2004
Lava	FPD	20	3	100	Raigrodski et al. 2006
DC-Zirkon	FPD	20	2	100	Vult von Steyern et al. 2005
Vitabloc Mark II	Inlays	66	5	94	Sjogren et al. 2004
ProCAD	Inlays, onlays	40	2	97	Guess et al. 2006
IPS Empress	Crowns	125	11	95.2	Fradeani et al. 2002
IPS Empress	Onlays	81	4	93	Naeselius et al 2008
IPS Empress 2	FDP	30	2	93	Esquivel –Upshaw et al. 2004
IPS Empress 2	Crowns	20	2	100	Taskonak et al. 2006
IPS e.max Press	Inlays, onlays	40	2	100	Guess et al. 2006
IPS e.max Press	FPD – crown retained FPD – inlay retained	36 45	4 3	100 89	Wolfart et al. 2005

CONCLUSION

Various all-ceramic restorations are now available including reinforced ceramic core systems (aluminium oxide and zirconium oxide) and resin-bonded ceramics (glass-ceramics). They can be processed in a number of different ways such as sintering, slip casting, hot pressing and CAD/CAM technology. As more systems are being introduced, it is imperative to understand the individual systems and factors that can influence their success. Recent

progress has involved the development of stronger, tougher all-ceramic materials and new enhanced processing systems are constantly evolving and improving such as CAD/CAM. Clinical trials are essential for understanding the all-ceramic systems, their modes of success, failure and limitations. Choice of material, clinical and manufacturing technique and patient selection are all paramount for success. The future of all-ceramics will certainly progress, with new materials constantly being developed in the search for the ultimate material regarding mechanical, physical and aesthetic properties.

REFERENCES

[1] Guazzato M, Albakry M, Ringer SP, Swain MV. Strength, fracture toughness and microstructure of a selection of all-ceramic materials. Part II. Zirconia-based dental ceramics. *Dent Mater.* 2004 Jun;20(5):449-56.

[2] Kelly JR, Nishimura I, Campbell SD. Ceramics in dentistry: historical roots and current perspectives. *The Journal of prosthetic dentistry.* 1996 Jan;75(1):18-32.

[3] Seha Mirkelam M, Pamuk S, Balkaya MC, Akgungor G. Effect of Tuf-Coat on Feldspathic porcelain materials. *Journal of oral rehabilitation*. 2005 Jan;32(1):39-45.

[4] van Dijken JW, Ormin A, Olofsson AL. Clinical performance of pressed ceramic inlays luted with resin-modified glass ionomer and autopolymerizing resin composite cements. *J Prosthet Dent*. 1999 Nov;82(5):529-35.

[5] Scherrer SS, Kelly JR, Quinn GD, Xu K. Fracture toughness (KIc) of a dental porcelain determined by fractographic analysis. *Dent Mater*. 1999 Sep;15(5):342-8.

[6] Barnfather KD, Brunton PA. Restoration of the upper dental arch using Lava all-ceramic crown and bridgework. *British dental journal. 2*007 Jun 23;202(12):731-5.

[7] Touati B, Miara P, Nathanson D. *Esthetic dentistry and ceramic restorations.* London: Martin Dunitz 1999.

[8] Callister W. *Materials Science and Engineering:* An introduction. 3rd ed. New York: John Wiley and Sons 1994.

[9] Van Noort R. *Introduction to Dental Materials:* Mosby 2002.

[10] Anusavice K. *Philip's Science of Dental Materials*. 11th ed. Philadelphia: Saunders 2003.

[11] Craig R. *Restorative dental materials*. 11th ed: St Louis, Mosby 2002.

[12] Fairhurst CW. *Dental ceramics: the state of the science. Advances in dental research*. 1992 Sep;6:78-81.

[13] McMillan P. *Glass-ceramics*. 2nd ed. London: Academic Press 1979.

[14] Kurdyk B. Nicolas Dubois de Chemant and the use of porcelain in dental art. *J Hist Dent.* 1999;47(3):126-8.

[15] Wildgoose DG, Johnson A, Winstanley RB. Glass/ceramic/refractory techniques, their development and introduction into dentistry: A historical literature review. *The Journal of prosthetic dentistry*. 2004 Feb;91(2):136-43.

[16] McLean JW, Hughes TH. The reinforcement of dental porcelain with ceramic oxides. *British dental journal*. 1965 Sep 21;119(6):251-67.

[17] Horn H. *Porcelain laminate veneers bonded to etched enamel*. Dental clinics of North America. 1983;27(4):671-84.

[18] Brecker S. Porcelain baked to gold: a new medium in prosthodontics. *The Journal of prosthetic dentistry*. 1956;6:801-10.
[19] Kelly JR. Ceramics in restorative and prosthetic dentistry. Annual Review of Materials *Science*. 1997;27:443-68.
[20] Piddock V, Qualtrough AJ. Dental ceramics—an update. *Journal of dentistry.* 1990 Oct;18(5):227-35.
[21] Denry I. Recent advances in ceramics for dentistry. *Crit Rev Oral Biol Med.* 1996;7(2):134-43.
[22] Mackert JR, Jr., Evans AL. Effect of cooling rate on leucite volume fraction in dental porcelains. *Journal of dental research*. 1991 Feb;70(2):137-9.
[23] Fischer H, Marx R. Improvement of strength parameters of a leucite-reinforced glass ceramic by dual-ion exchange. *Journal of dental research*. 2001 Jan;80(1):336-9.
[24] Denry IL, Holloway JA, Rosenstiel SF. Effect of ion exchange on the microstructure, strength, and thermal expansion behavior of a leucite-reinforced porcelain. *Journal of dental research*. 1998 Apr;77(4):583-8.
[25] Wassell RW, Walls AW, Steele JG. Crowns and extra-coronal restorations: materials selection. *British dental journal.* 2002 Feb 23;192(4):199-202, 5-11.
[26] Fischer H, Maier HR, Marx R. Improved reliability of leucite reinforced glass by ion exchange. *Dent Mater.* 2000 Mar;16(2):120-8.
[27] Anusavice KJ, Hojjatie B, Chang TC. Effect of grinding and fluoride-gel exposure on strength of ion-exchanged porcelain. *Journal of dental research.* 1994 Aug;73(8):1444-9.
[28] Seghi RR, Crispin BC, Mito W. The effect of ion exchange on the flexural strength of feldspathic porcelains. *The International journal of prosthodontics.* 1990 Mar-Apr;3(2):130-4.
[29] Seghi RR, Denry I, Brajevic F. Effects of ion exchange on hardness and fracture toughness of dental ceramics. *The International journal of prosthodontics*. 1992 Jul-Aug;5(4):309-14.
[30] Griggs JA, Thompson JY, Anusavice KJ. Effects of flaw size and auto-glaze treatment on porcelain strength. *Journal of dental research.* 1996 Jun;75(6):1414-7.
[31] Anusavice KJ, Hojjatie B. Effect of thermal tempering on strength and crack propagation behavior of feldspathic porcelains. *Journal of dental research*. 1991 Jun;70(6):1009-13.
[32] Giordano RA, 2nd, Pelletier L, Campbell S, Pober R. Flexural strength of an infused ceramic, glass ceramic, and feldspathic porcelain. *The Journal of prosthetic dentistry.* 1995 May;73(5):411-8.
[33] Jung YG, Peterson IM, Pajares A, Lawn BR. Contact damage resistance and strength degradation of glass-infiltrated alumina and spinel ceramics. *Journal of dental research.* 1999 Mar;78(3):804-14.
[34] Hwang JW, Yang JH. Fracture strength of copy-milled and conventional In-Ceram crowns. *Journal of oral rehabilitation.* 2001 Jul;28(7):678-83.
[35] Raigrodski AJ. Contemporary materials and technologies for all-ceramic fixed partial dentures: a review of the literature. *The Journal of prosthetic dentistry.* 2004 Dec;92(6):557-62.

[36] Wagner WC, Chu TM. Biaxial flexural strength and indentation fracture toughness of three new dental core ceramics. *The Journal of prosthetic dentistry.* 1996 Aug;76(2):140-4.
[37] Albakry M, Guazzato M, Swain MV. Fracture toughness and hardness evaluation of three pressable all-ceramic dental materials. *Journal of dentistry.* 2003 Mar;31(3):181-8.
[38] Seghi RR, Sorensen JA. Relative flexural strength of six new ceramic materials. *The International journal of prosthodontics.* 1995 May-Jun;8(3):239-46.
[39] Della Bona A, Donassollo TA, Demarco FF, Barrett AA, Mecholsky JJ, Jr. Characterization and surface treatment effects on topography of a glass-infiltrated alumina/zirconia-reinforced ceramic. *Dent Mater.* 2007 Jun;23(6):769-75.
[40] Seghi RR, Denry IL, Rosenstiel SF. Relative fracture toughness and hardness of new dental ceramics. *The Journal of prosthetic dentistry.* 1995 Aug;74(2):145-50.
[41] Tinschert J, Zwez D, Marx R, Anusavice KJ. Structural reliability of alumina-, feldspar-, leucite-, mica- and zirconia-based ceramics. *Journal of dentistry.* 2000 Sep;28(7):529-35.
[42] Guazzato M, Albakry M, Quach L, Swain MV. Influence of surface and heat treatments on the flexural strength of a glass-infiltrated alumina/zirconia-reinforced dental ceramic. *Dent Mater.* 2005 May;21(5):454-63.
[43] Heffernan MJ, Aquilino SA, Diaz-Arnold AM, Haselton DR, Stanford CM, Vargas MA. Relative translucency of six all-ceramic systems. Part I: core materials. *The Journal of prosthetic dentistry.* 2002 Jul;88(1):4-9.
[44] Eidenbenz S, Lehner CR, Scharer P. Copy milling ceramic inlays from resin analogs: a practicable approach with the CELAY system. The International journal of prosthodontics. 1994 Mar-Apr;7(2):134-42.
[45] Goldstein RE, Garber DA, Goldstein CE, Schwartz CG, Salama MA, Gribble AR, et al. Esthetic update: the changing esthetic dental practice. *Journal of the American Dental Association* (1939). 1994 Nov;125(11):1447-56.
[46] Guazzato M, Albakry M, Ringer SP, Swain MV. Strength, fracture toughness and microstructure of a selection of all-ceramic materials. Part I. Pressable and alumina glass-infiltrated ceramics. *Dent Mater*. 2004 Jun;20(5):441-8.
[47] Rimmer S. Modern dental ceramics: an overview. *International Dentistry SA*. 2006;1(3):66-72.
[48] Valandro LF, Della Bona A, Antonio Bottino M, Neisser MP. The effect of ceramic surface treatment on bonding to densely sintered alumina ceramic. *The Journal of prosthetic dentistry.* 2005 Mar;93(3):253-9.
[49] Casson AM, Glyn Jones JC, Youngson CC, Wood DJ. The effect of luting media on the fracture resistance of a flame sprayed all-ceramic crown. *Journal of dentistry*. 2001 Nov;29(8):539-44.
[50] Ardlin BI. Transformation-toughened zirconia for dental inlays, crowns and bridges: chemical stability and effect of low-temperature aging on flexural strength and surface structure. *Dent Mater*. 2002 Dec;18(8):590-5.
[51] Piconi C, Maccauro G. Zirconia as a ceramic biomaterial. *Biomaterials.* 1999 Jan;20(1):1-25.

[52] Curtis AR, Wright AJ, Fleming GJ. The influence of surface modification techniques on the performance of a Y-TZP dental ceramic. *Journal of dentistry*. 2006 Mar;34(3):195-206.
[53] Pittayachawan P, McDonald A, Petrie A, Knowles JC. The biaxial flexural strength and fatigue property of Lava Y-TZP dental ceramic. *Dent Mater.* 2007 Aug;23(8):1018-29.
[54] Sundh A, Sjogren G. Fracture resistance of all-ceramic zirconia bridges with differing phase stabilizers and quality of sintering. *Dent Mater.* 2006 Aug;22(8):778-84.
[55] Awliya W, Oden A, Yaman P, Dennison JB, Razzoog ME. Shear bond strength of a resin cement to densely sintered high-purity alumina with various surface conditions. *Acta odontologica Scandinavica*. 1998 Feb;56(1):9-13.
[56] Derand P, Derand T. Bond strength of luting cements to zirconium oxide ceramics. *The International journal of prosthodontics.* 2000 Mar-Apr;13(2):131-5.
[57] Madani M, Chu FC, McDonald AV, Smales RJ. Effects of surface treatments on shear bond strengths between a resin cement and an alumina core. *The Journal of prosthetic dentistry.* 2000 Jun;83(6):644-7.
[58] Blatz MB, Sadan A, Kern M. Resin-ceramic bonding: a review of the literature. *The Journal of prosthetic dentistry.* 2003 Mar;89(3):268-74.
[59] Kern M, Strub JR. Bonding to alumina ceramic in restorative dentistry: clinical results over up to 5 years. *Journal of dentistry.* 1998 Mar;26(3):245-9.
[60] Fischer J, Stawarczyk B, Tomic M, Strub JR, Hammerle CH. Effect of thermal misfit between different veneering ceramics and zirconia frameworks on in vitro fracture load of single crowns. *Dental materials journal.* 2007 Nov;26(6):766-72.
[61] Aboushelib MN, de Kler M, van der Zel JM, Feilzer AJ. Effect of veneering method on the fracture and bond strength of bilayered zirconia restorations. *The International journal of prosthodontics.* 2008 May-Jun;21(3):237-40.
[62] Burke FJ. Fracture resistance of teeth restored with dentin-bonded crowns: the effect of increased tooth preparation. *Quintessence Int.* 1996 Feb;27(2):115-21.
[63] Filho AM, Vieira LC, Araujo E, Monteiro Junior S. Effect of different ceramic surface treatments on resin microtensile bond strength. *J Prosthodont.* 2004 Mar;13(1):28-35.
[64] Hooshmand T, van Noort R, Keshvad A. Bond durability of the resin-bonded and silane treated ceramic surface. *Dent Mater.* 2002 Mar;18(2):179-88.
[65] Aida M, Hayakawa T, Mizukawa K. Adhesion of composite to porcelain with various surface conditions. *The Journal of prosthetic dentistry.* 1995 May;73(5):464-70.
[66] Ozcan M, Vallittu PK. Effect of surface conditioning methods on the bond strength of luting cement to ceramics. *Dent Mater*. 2003 Dec;19(8):725-31.
[67] Della Bona A, Shen C, Anusavice KJ. Work of adhesion of resin on treated lithia disilicate-based ceramic. *Dent Mater.* 2004 May;20(4):338-44.
[68] Soderholm KJ, Shang SW. Molecular orientation of silane at the surface of colloidal silica. *Journal of dental research.* 1993 Jun;72(6):1050-4.
[69] Fleming GJ, Maguire FR, Bhamra G, Burke FM, Marquis PM. The strengthening mechanism of resin cements on porcelain surfaces. *Journal of dental research.* 2006 Mar;85(3):272-6.
[70] Burke FJ. Maximising the fracture resistance of dentine-bonded all-ceramic crowns. *Journal of dentistry.* 1999 Mar;27(3):169-73.
[71] Marquis P. The influence of cements on the mechanical performance of dental ceramics. *Bioceramics*. 1992;5:317-24.

[72] Nathanson D, Riis D. Advances and current research in ceramic restorative materials. *Current opinion in cosmetic dentistry.* 1993:34-40.
[73] Strnad Z. *Glass ceramic materials*. Amsterdam: Elsevier Science 1986.
[74] Varshneya A. *Fundamentals of Inoganic Glasses*. New York: Academic Press 1994.
[75] DeJong B, Adams J, Aitken B, Dickinson J, *Fine G. Glass Ceramics*: Wiley - VCH Verlag GmbH & Co. Weinheim 2005.
[76] Grossman D, Walters H. The chemical durability of dental ceramics. *Journal of dental research.* 1989;63:574: 234.
[77] Kelsey WP, 3rd, Cavel T, Blankenau RJ, Barkmeier WW, Wilwerding TM, Latta MA. 4-year clinical study of castable ceramic crowns. *American journal of dentistry*. 1995 Oct;8(5):259-62.
[78] Holand W, Rheinberger V, Apel E, van 't Hoen C, Holand M, Dommann A, et al. Clinical applications of glass-ceramics in dentistry. *Journal of materials science*. 2006 Nov;17(11):1037-42.
[79] Giordano R. Materials for chairside CAD/CAM-produced restorations. *Journal of the American Dental Association* (1939). 2006 Sep;137 Suppl:14S-21S.
[80] [Otto T. [Cerec restorations. *Cerec inlays and onlays: the clinical results and experiences after 6 years of use in private practice]*. Schweizer Monatsschrift fur Zahnmedizin = Revue mensuelle suisse d'odonto-stomatologie = Rivista mensile svizzera di odontologia e stomatologia / SSO. 1995;105(8):1038-46.
[81] Gaglio MA. Esthetic restorations designed with confidence and predictability. *Compend Contin Educ Den*t. 2001 Jun;22(6 Suppl):30-4.
[82] IvoclarVivadentAG. *Scientific documentation ProCAD*. 2002.
[83] Wohlwend A, Scharer P, Stub JR. [Metalloceramic and full ceramic restorations (1]. *Die Quintessenz*. 1990 Jun;41(6):981-91.
[84] Albakry M, Guazzato M, Swain MV. Biaxial flexural strength, elastic moduli, and x-ray diffraction characterization of three pressable all-ceramic materials. *The Journal of prosthetic dentistry.* 2003 Apr;89(4):374-80.
[85] Holand W, GH B. *Glass-ceramic technology*. Columbus, Ohio: American Ceramics Society 2002.
[86] Cattell MJ, Palumbo RP, Knowles JC, Clarke RL, Samarawickrama DY. The effect of veneering and heat treatment on the flexural strength of Empress 2 ceramics. *Journal of dentistry.* 2002 May;30(4):161-9.
[87] Dong JK, Luthy H, Wohlwend A, Scharer P. Heat-pressed ceramics: technology and strength. *The International journal of prosthodontics*. 1992 Jan-Feb;5(1):9-16.
[88] Holand W, Schweiger M, Frank M, Rheinberger V. A comparison of the microstructure and properties of the IPS Empress 2 and the IPS Empress glass-ceramics. *Journal of biomedical materials research.* 2000;53(4):297-303.
[89] IvoclarVivadent. *IPS Empress System. Scientific Documentation*. 2007.
[90] [Buhler-Zemp P, Volkel T. Scientific documentation IPS e.max CAD. *Schaan, Leichtenstein: Ivoclar Vivadent* AG 2005.
[91] Kingery W, Bowen H, Uhlmann D. *Introduction to Ceramics*. 2nd ed. New York: John Wiley and Sons 1976.
[92] Jones D, Wilson H. Some properties of dental ceramics. *Journal of oral rehabilitation.* 1975;2:379-96.

[93] Magne P. Composite resins and bonded porcelain: the postamalgam era? *Journal of the California Dental Association.* 2006 Feb;34(2):135-47.
[94] Gorman CM, McDevitt WE, Hill RG. Comparison of two heat-pressed all-ceramic dental materials. *Dent Mater.* 2000 Nov;16(6):389-95.
[95] Oh SC, Dong JK, Luthy H, Scharer P. Strength and microstructure of IPS Empress 2 glass-ceramic after different treatments. *The International journal of prosthodontics*. 2000 Nov-Dec;13(6):468-72.
[96] Yeo IS, Yang JH, Lee JB. In vitro marginal fit of three all-ceramic crown systems. *The Journal of prosthetic dentistry*. 2003 Nov;90(5):459-64.
[97] Fasbindel D. Clinical performance of chairside CAD/CAM restorations. *JADA*. 2006;137:22S-31S.
[98] Mormann WH, Brandestini M, Lutz F. [The Cerec system: computer-assisted preparation of direct ceramic inlays in 1 setting]. *Die Quintessenz*. 1987 Mar;38(3):457-70.
[99] Mormann WH. *The evolution of the CEREC system. JADA*. 2006;137:7S-13S.
[100] Strub JR, Rekow ED, Witkowski S. Computer-aided design and fabrication of dental restorations. *JADA*. 2006;137:1289-96.
[101] Martin N, Jedynakiewicz NM. Clinical performance of CEREC ceramic inlays: a systematic review. *Dent Mater.* 1999 Jan;15(1):54-61.
[102] Sjogren G, Bergman M, Molin M, Bessing C. A clinical examination of ceramic (Cerec) inlays. *Acta odontologica Scandinavica.* 1992 Jun;50(3):171-8.
[103] Dunn M. Biogeneric and user-friendly: the Cerec 3D software upgrade V3.00. *International journal of computerized dentistry.* 2007 Jan;10(1):109-17.
[104] Conrad HJ, Seong WJ, Pesun IJ. Current ceramic materials and systems with clinical recommendations: a systematic review. *The Journal of prosthetic dentistry*. 2007 Nov;98(5):389-404.
[105] Marquardt P, Strub JR. Survival rates of IPS empress 2 all-ceramic crowns and fixed partial dentures: results of a 5-year prospective clinical study. *Quintessence Int*. 2006 Apr;37(4):253-9.

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Chapter 2

DEVELOPMENT OF LOW-SHRINKAGE DENTAL COMPOSITES IN DENTISTRY: A REVIEW OF THE TRENDS AND TECHNIQUES APPLIED

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ABSTRACT

Dental composite resins have revolutionized modern clinical dentistry. They are widely used for restoring teeth and cosmetic dentistry due to their esthetic and handling properties. Despite their wide applications, present day composite resins shrink when cured. This polymerization shrinkage generate stresses which affect the marginal seal between the tooth/restoration interfaces leading to secondary caries, post-operative sensitivity, tooth fracture, bond failure and marginal leakage. Other problems associated with current dental composites include inadequate wear resistance and the leaching of uncured organic monomers. The development of low shrinkage resins is therefore an important research focus in dentistry and remains a challenge. In this review, different polymerization techniques such as soft-start, pulse cure and pulse delay used to minimize shrinkage clinically will be discussed. The effect of the different light-curing techniques on the crosslink density of composites will also be reported. Recent developments of low shrinkage composites including some of our work on silsesquioxane in the laboratory will also be highlighted.

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INTRODUCTION

The modernization of clinical dentistry evolved from the development of light-activated dental composite resins. They are viewed as an attractive alternative to amalgam fillings and have superseded chemically cured counterparts. What are light-activated dental composites? What are their superior advantages over chemically cured composite resins and dental amalgam?

Composite materials refer to a mixture of two or more distinctly different materials with properties that are superior or intermediate to those of the individual constituents. Light-activated dental composites are tooth-coloured filling materials made up of synthetic polymers such as 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]-propane (Bis-GMA) / triethyleneglycol dimethacrylate (TEGDMA) (most commonly used resins) (Figure 1), inorganic particulate fillers, initiators and activators, silane coupling agents (for bonding of the fillers to the matrix), pigments and stabilizers [1]. They undergo polymerization upon blue light-irradiation in the region of 410-500 nm to form a cross-linked polymer network. Light in this region is absorbed efficiently by photoinitiators usually camphorquione (CQ), which creates an excited state that reacts with an amine reducing agent (activators) such as *N,N*-dimethylaminoethyl methacrylate (DMAEMA) or ethyl *p*-dimethylaminobenzoate (DMAB) to produce free radicals for the polymerization of the organic matrix (Figure 2) [2, 3].

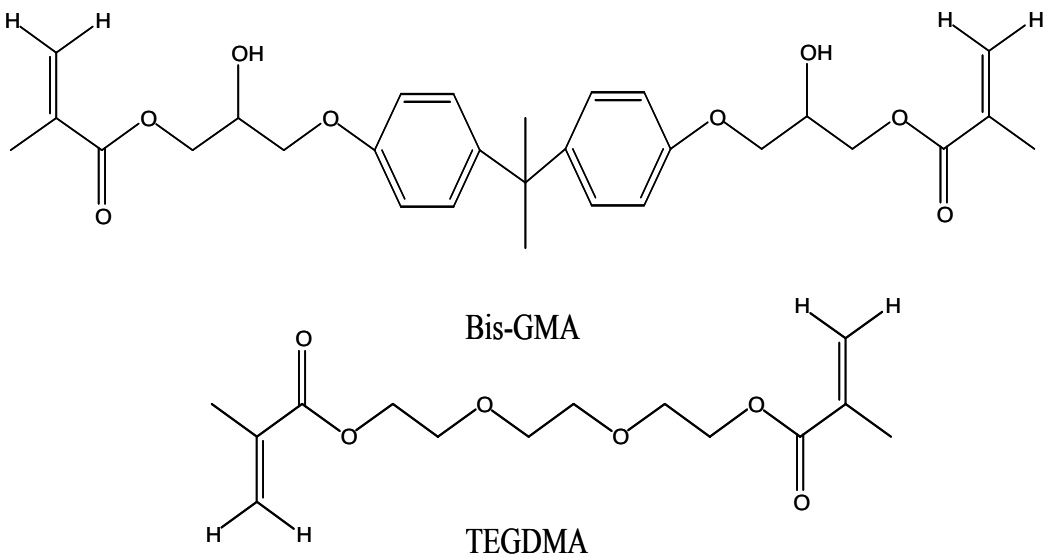


Figure 1. Chemical structure of conventional dental monomers, bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]-propane (Bis-GMA) / triethyleneglycol dimethacrylate (TEGDMA).

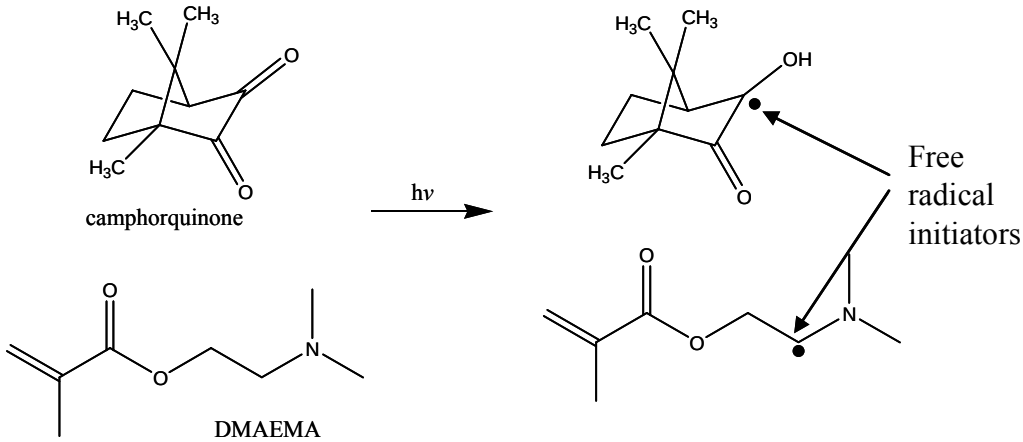


Figure 2. Light activation mechanism of camphorquinone with an amine reducing agent, *N,N*-dimethylaminoethyl methacrylate (DMAEMA).

When compared to chemically cured composites, light-activated composite resins offer the advantages of higher wear resistance, lower thermal expansion, lower polymerization shrinkage and controlled working time where clinicians have the freedom to time their polymerization thus getting rid of the time consuming blending procedures that often introduce unnecessary porosities such as oxygen to the restorations as observed in chemically cured composites. Chemically cured composites also known as self or auto cured composites, involved the blending of two pastes that brought the monomers matrix and the initiator (dibenzoyl peroxide) / activator (*N,N*-di-(2-hydroxyethyl)-*p*-toluidine (DHEPT) or *N,N*-dimethy-*p*-toluidine (DMPT)) system for the initiation of the cross-linking reactions. The lack of wear resistance and strength prevented them from preserving restoration contour in areas subject to abrasion or attrition and also affected their use in high-stress areas due to flowability of the materials under load. Besides that, self-cured composites were also found to be associated with high polymerization shrinkage and coefficient of thermal expansion which led to problems such as microleakage and discoloration at the margins due to percolation [4]. Furthermore, clinicians were also restricted by the polymerization setting time when placing and shaping the restorations. Clinical studies conducted have also showed that over a period of time, restorations that were restored with chemically-cured composites tend to undergo more darkening when compared to light-activated composites [5]. Thus, the aforementioned limitations of self-cured composites have led to the development of light-activated ones.

Besides the superior properties displayed by light-activated resins when compared to chemically cured composites, tooth-coloured composite resins were also viewed as an attractive amalgam substitution due to concerns over mercury toxicity and increasing aesthetic demands by patients and clinicians [6, 7]. Thus, modern day composites are widely used for restorations in the anterior and posterior regions, indirect inlays and onlays, pit and fissure sealants as well as crowns and implants.

The development of light-activated composites to date has progress rapidly with major improvements made in the area of increased filler loading along with variation in distribution, size, shape and composition. Modifications to the filler components bring improvements in wear resistance, color stability, strength, radiopacity and degree of conversion of dental

composites and thus the overall improvement in clinical performance of these materials. However, despite vast improvements in composite materials and their mechanical properties, present day composite resins still have shortcomings limiting their application. Inadequate resistance to wear (loss of anatomic form) under masticatory attrition, fracture of restorations, incomplete conversion and cross-linking of the organic matrix, discoloration, marginal adaptation, secondary caries and marginal leakage due to polymerization shrinkage are some of the factors limiting the longevity of composite resins [8, 9].

LIMITATIONS OF CURRENT DENTAL COMPOSITES

Commercial dental composites exhibit 2–14% volumetric shrinkage during the polymerization process [10-12] and are affected by factors such as constituents of the resin-based composite material, configuration of the cavity preparation, spectral and power distribution of the visible light-curing unit, and finally clinical technique [13]. When composites shrink, stresses are generated at the composite/tooth interface. These shrinkage stresses can cause marginal openings if the bonding system is unable to withstand the polymerization forces and thus lead to leakage and ultimately caries. Despite the dramatic improvements in the formulation of newer generation bonding agents with enhanced marginal adaptation and bond strengths, the goal of a perfect marginal seal is still not achievable. Clinical studies carried out for resin-based composite restorations for Class I and II cavities for a period of 3 to 6 years have also shown that secondary and/or recurrent caries were the main reasons for restoration failure [8, 14-16] and polymerization shrinkage has been cited as one of the most significant factors influencing the seal between tooth structure and polymer-based restorative materials.

All composites shrink upon light-activation and the total shrinkage of composite materials can be divided into pre-gel and post-gel phases. During the pre-gel polymerization, the composite is able to flow and stresses within the structure are relieved [17]. After gelation, viscosity increases significantly and stresses due to shrinkage cannot be compensated. Post-gel polymerization thus results in significant stresses in the surrounding tooth structure and composite tooth bond [18] that may lead to bond failure, microleakage, post-operative sensitivity and recurrent caries. These stresses could also result in deformation of the surrounding tooth structure if the composite-tooth bond is strong, causing the tooth to fracture [19].

As aforementioned, the stress associated with polymerization shrinkage is one of the most significant problems associated with current composite materials as it adversely affects the seal at the composite/tooth interface which led to the occurrence of secondary caries [20]. When bonding of the adhesive to the tooth structure is inadequate, composite shrinks and pulls away from the cavity walls, forming an opening. This opening at the restoration interface leads to clinical problems such as microleakage, straining, sensitivity, and/or recurrent caries. However, when the bonding to tooth structure is strong enough, polymerization stress is applied to the tooth as composites shrink which leads to problems such as fractured cusps, movement of cusps, and/or postoperative sensitivity [13].

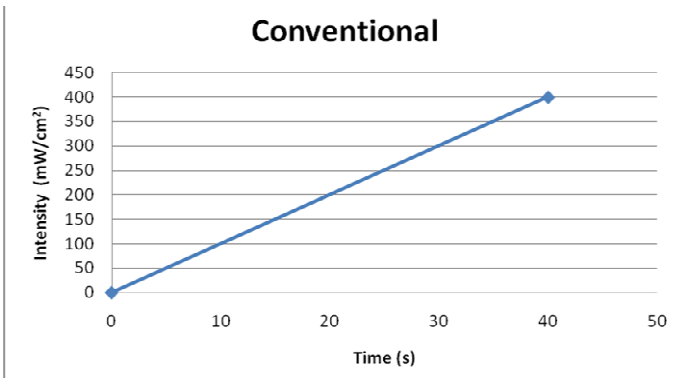
Besides, differences in monomer chemistry, various degrees of final polymerization, filler types and filler concentrations, the amount of stress generated due to polymerization

shrinkage is also dependent on the configuration of the cavity preparation by clinicians. Configuration factor, commonly known as the C-factor, is defined as the ratio of the bonded area of the restoration to the unbonded area [18]. High C-factor are often associated with a higher stress on the bonded surfaces [21]. Since composite flow is more likely to occur from the free surfaces of the specimen, a higher proportion of free composite surface would correspond to a smaller restriction to shrinkage, thereby reducing stress. When the free surface is reduced, the ability to flow and compensate for shrinkage is restricted by the bonded surfaces thus, increasing stress. As cavity preparations present a much more complex geometry with heterogeneous stress distribution [22], the application of the C-factor concept to clinical practice must be performed carefully by the clinicians in order to minimize shrinkage and its accompanying stress.

Besides C-factor, other clinical techniques used for minimizing polymerization shrinkage and its accompanying stresses includes incremental layering of composite during placement [23] and application of a low elastic modulus liner as a stress absorber between the tooth and shrinking composite restorative [24]. One recent method which has gained popularity over the years among researchers and clinicians for the reduction of polymerization shrinkage without affecting the degree of conversion in light-activated composites is to reduce the viscosity during setting by means of controlled polymerization.

CONTROLLED POLYMERIZATION

Controlled polymerization refers to the various curing techniques employed by clinicians and researchers for the reduction of polymerization shrinkage and its accompanying stresses. In contrast to the conventional continuous curing method, controlled polymerization set to reduce shrinkage by slowing down the rate of polymerization. Various controlled polymerization techniques include curing with an intensity that increases exponentially from low to high (ramp/ exponential), application of short pulses of energy (pulse activation), delays between exposures (pulse delay) or pre-polymerization at low-intensity light followed by a final cure at high intensity (soft-start techniques) (Figure 3). It was postulated that with controlled polymerization, clinicians can reduce polymerization shrinkage and stresses of various materials by timing and controlling their polymerization more efficiently. The delay, dark period and low-intensity curing associated with the various curing modes allowed for composite flow to take place by delaying the gel-point. Gel-point refers to the transformation of the composites from a viscous state to a rigid solid while composite flow is defined as the capability of the molecules to go into a new position before cross-linking. Thus the low modulus developed by the composites in the early polymerization phase of the controlled curing techniques helps in relieving stress through composite flow and finally the reduction of polymerization shrinkage. In recent years, there have been tremendous studies and research carried out to determine the effectiveness of reducing polymerization shrinkage and stresses through controlled polymerization light curing techniques.



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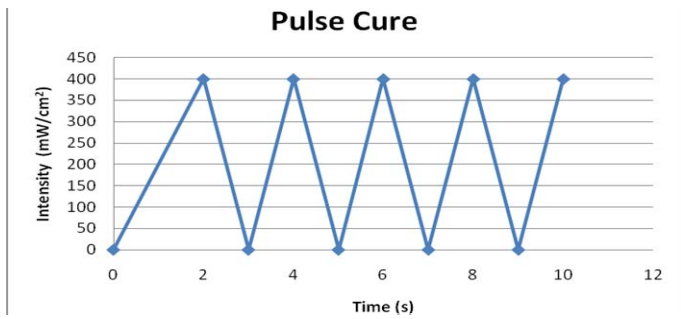

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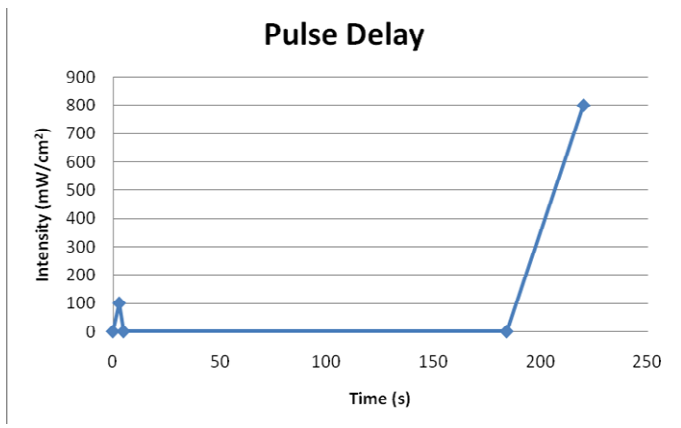

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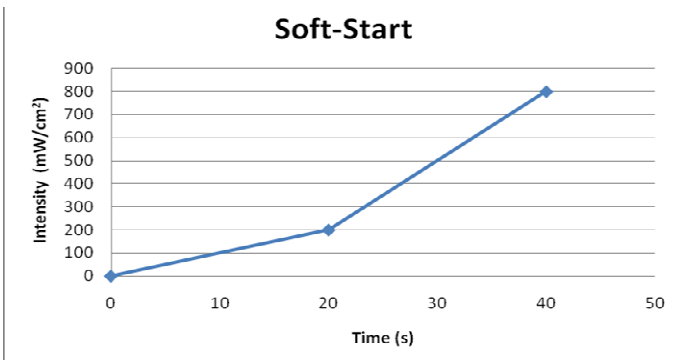

(d)

Figure 3. Examples of the different light-curing profiles (a) conventional, (b) pulse cure, (c) pulse delay, (d) soft-start used for polymerization of dental composites.

Uno & Asmussen [25], Goracci et al. [26] and Koran & Kürschner [27] have demonstrated through studies in the 90s that marginal gap reduction, better marginal adhesion were observed in composite cured through a sequential irradiation approach when compared to the continuous irradiation mode. In addition, Koran & Kürschner [27] have also demonstrated that even though the degree of conversion and final shrinkage did not differ significantly with either approach when the total light energy density applied was kept constant, the dynamic of shrinkage were found to be superior with the two step approach. In 2002, Lim et al. [28] demonstrated that curing composites using the pulse delay mode helps to reduce polymerization contraction stresses. No significant reduction in polymerization stresses was observed when composites were cured using the soft-start approach. Results obtained correlate well to that of Friedl et al. [29] and Bouschlicher et al. [30] where marginal adaption and shrinkage forces of composites were not influenced by either the conventional or two step curing modes. Thus, the introduction of a gap period between pulses is important for enhancement of composite flow which helps in the reduction of composite shrinkage and stresses [31, 32].

While studies [27, 28, 33, 34] have shown that curing of composites using soft-start technique did not result in significant reduction in shrinkage and stresses when compared to continuous mode, others have found that controlled polymerization helps to reduce gap formation [35-37], microleakage [38, 39], polymerization shrinkage and stresses [32, 40-42] without affecting their degree of conversion and mechanical properties. Thus, their use in clinical practice has been encouraged by Alonso et al. [35].

In order to evaluate the usefulness of controlled polymerization in clinical practice, we have also carried out studies to determine the effectiveness of different light-curing modes on composite cure and shrinkage [43, 44]. In the studies, it was found that the use of pulse delay mode helps to reduce shrinkage when compared to the continuous mode. However, the technique also results in lower bottom hardness value when cured on a 2 mm think specimens. In order to have a fair comparison, we conducted another study to evaluate the different light curing regimens at a fixed light energy density [45]. It was found that pulse delay and soft-start curing techniques does help to decrease post-gel polymerization shrinkage when compared to the conventional light-curing mode. The effectiveness of cure may also be enhanced by the use of soft-start curing technique [46]. It was also observed that the use of soft-start and pulse activation modes of some light curing units (Halogen vs. LED (light-emitting diodes)) may help in the reduction of post-gel shrinkage [10]. Thus, the reduction of polymerization shrinkage of composites was found to be not only materials dependent, mode dependent but also light curing unit dependent.

While studies on the usefulness of controlled polymerization for the reduction of polymerization shrinkage and stresses remains to be debatable, Asmussen & Peutzfeldt [47, 48] discovered that the use of pulse delay and soft-start modes resulted in a different polymer structure compared to composite cured using the conventional continuous mode despite similar degree of cure. In the study, it was postulated that the use of a continuous mode resulted in a more crosslink structure due to its ability to initiate more camphoroquinone and thus the formation of more growth centers compared to the pulse delay and soft-start modes where fewer growth centers were formed as a result of lower and shorter initial curing. Thus, polymerization using the controlled curing technique (pulse delay and soft-start) resulted in a more linear polymer structure. The linear polymer structure obtained as a result of pulse delay and soft-start curing causes an increased susceptibility to softening in ethanol. The results

obtained suggest that composites cured by controlled polymerization techniques may be more susceptible to softening by food and beverages and also an increased in free monomer leaching. While pulse delay and soft-start techniques have been found to be useful for the reduction of marginal gap and polymerization shrinkage and its accompanying stress, the use of these techniques may become less attractive in the dental community. However, studies in this aspect are still limited.

In order to evaluate the aforementioned results further, we conducted a study to evaluate the crosslinking density of composite cured with both the conventional and controlled polymerization techniques under the condition whereby constant light energy density were applied for all curing modes [49]. In this study, the degree of crosslinking density was measured both directly and indirectly through measurements of the glass transition temperature and hardness after ethanol storage, respectively. The glass transition temperature (T_g) associated with the controlled polymerization curing modes in this study was found to be lower than the conventional cure mode. As T_g values of a polymer system are more dependent upon crosslinking, the lower T_g values obtained for the controlled polymerization techniques suggest less crosslinking. Results obtained in this study also suggest that polymerization with pulse delay mode resulted in a lower crosslink density and gave rise to polymers with an increased susceptibility to softening in ethanol. However, when we investigated the amount of uncured monomers that leached out of the composite when cured by the different light-curing techniques, we found that leaching of the monomers are light-curing units dependent rather than polymer structures or light-curing modes dependent [50]. While the usefulness of controlled polymerization for the reduction of polymerization shrinkage remains an elusive target, one of the greatest challenges and the ultimate solution to polymerization shrinkage is to develop expanding, low-shrinking or non-shrinking resins.

DEVELOPMENT OF LOW SHRINKING COMPOSITES

While shrinkage stresses can be reduced by increasing filler loading, the ultimate solution to polymerization shrinkage is to develop “non-shrinking” resins. Although earlier efforts to synthesize such resins were not successful, several developments in the last decade are more encouraging.

RING-OPENING MONOMERS

In order to reduce shrinkage, expanding monomers such as spiro-orthocarbonates (SOCs) derivatives have been synthesized by Stansbury [51,52] in 1992. These SOCs monomers expand during polymerization through “a double-ring opening process”. The SOCs derivatives were attached with polymerizable group such as the methylene (Figure 4) and the methacrylate (Figure 5) group to enable cross-linking to take place upon curing. The first series of SOCs synthesized base on the methylene side chain was found to have slow reactivity when compared to the SOCs compounds with methacrylate as side chain. The methacrylate substituted SOCs were found to be very reactive when polymerized in dilute

solutions with almost complete ring opening of the SOCs compounds. However, reactivity decreases with bulk curing and hence resulted in 1 % shrinkage.

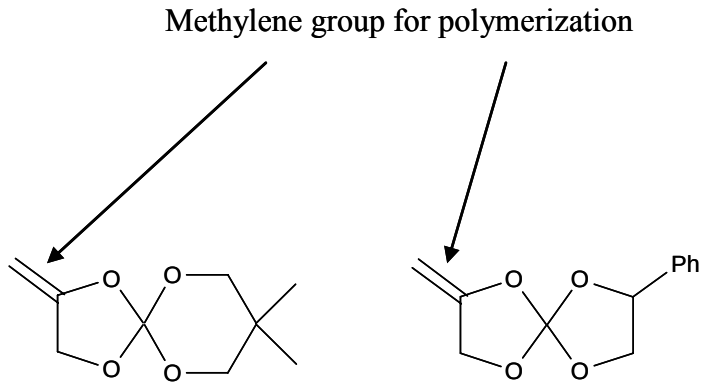

Figure 4. Examples of the different spiro-orthocarbonates (SOCs) containing methylene groups synthesized as ring-opening monomers.

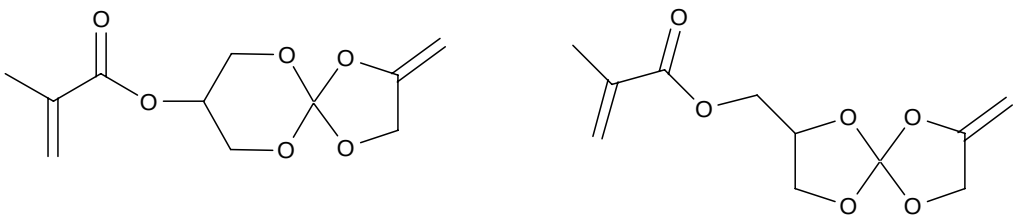

Figure 5. Chemical structures of spiro-orthocarbonate substituted methacrylate.

Besides the above mentioned compounds, a six-membered SOCs (trans/trans-2,3,8,9-di(tetramethylene)-1,5,7,11-tetraoxaspiro[5.5] undecane) (Figure 6) which exhibited a 3.5 % volume expansion when polymerized with cationic initiators such as (4-octyloxyphenyl)-phenyliodonium hexafluoroantimonate, has also been synthesized [53, 54]. When used as copolymers, results showed that the addition of as little as 5 % of the SOC monomers in an epoxy base produced a compound with substantial tensile strength and modulus, acceptable water sorption and solubility, and a slight expansion. While results of these studies are promising, no commercial materials are available to date. This may be due to the high cost of the monomer.

Further to SOCs, synthesis of SOEs (spiro ortho esthers) derivatives has also been reported by Miyazaki *et al.* [55]. These SOEs which contained polymerizable group such as the acrylate and methacrylate can undergo heat, ionic and free radical polymerization. However, the composites after polymerization were found to be low in strength without much reduction in shrinkage.

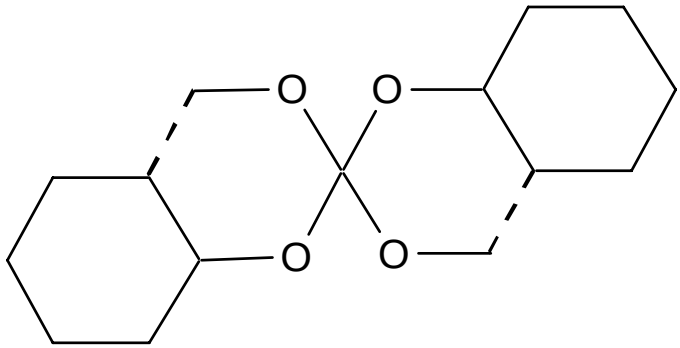

Figure 6. A cationic polymerizable spiro-orthocarbonate, trans/trans-2,3,8,9-di(tetramethylene)-1,5,7,11-tetraoxaspiro[5.5] undecane.

Besides SOCs, cationic photopolymerizable epoxy monomer such as cycloaliphatic epoxy is another class of compounds developed. These epoxy monomers can be polymerized using blue light through a three component initiating system which is made up of cationic initiators, camphorquinone and an amine activator. When compared to dental methacrylate, the cycloaliphatic epoxy resulted in a lower shrinkage. One example in the application of the epoxy resins is that of epoxy-polyol mixtures. Tilbrook *et al.* [56] has demonstrated through their study that with the correct choice of polyol and ratio of epoxy to polyol groups, a low shrinkage composite with comparable strength and stiffness can be obtained. However, the use of epoxy-polyol was not encouraged due to its high water sorption (polyol is hydrophilic in nature) which leads to cracking. One other area of development for the cycloaliphatic epoxy monomers is the development of Siloranes^{TM} which will be discussed further in the later part of this review.

In addition to epoxy resins, ring opening monomers such as oxetanes [57, 58], cyclic acetals [59, 60], cyclic allyl sulphides [61] and vinylcyclopropanes [62, 63] have also been evaluated for dental applications. However, none of them were found to be promising. While oxetanes has a higher basicity with the reactivity of polymerization being atmosphere dependent (more reactive in nitrogen) [58], cyclic acetals, cyclic allyl sulfides and vinylcyclopropanes compounds were found to be either unstable, have low reactivity, exhibited glass transition temperature (T_g) that were unacceptable for dental applications or resulted in polymers that have high flexibility.

LIQUID CRYSTALLINE MONOMERS

As the search for low-shrinking polymers continue, liquid crystalline or highly branched molecules have also been synthesized and evaluated for their usefulness as low-shrinking monomers. While liquid crystalline monomers have the advantages of low viscosity, high degree of conversion and low shrinkage properties, they were found to have melting temperatures greater than 80 ^{o}C making polymerization difficult. In an effort to overcome this problem, several new liquid crystalline dimethacrylates [64, 65] (Figure 7) and/or branched liquid crystalline bismethacrylates [66, 67] (Figure 8) monomers with polymerization shrinkage ranging from 1.3 – 2.5 vol% have been synthesized through chain modifications.

While these monomers resulted in low polymerization shrinkage, their mechanical properties were not promising due to the more flexible network.

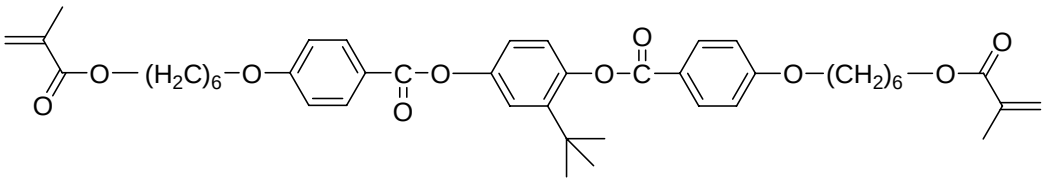

Figure 7. Example of near room temperature polymerizable liquid crystalline dimethacrylate.

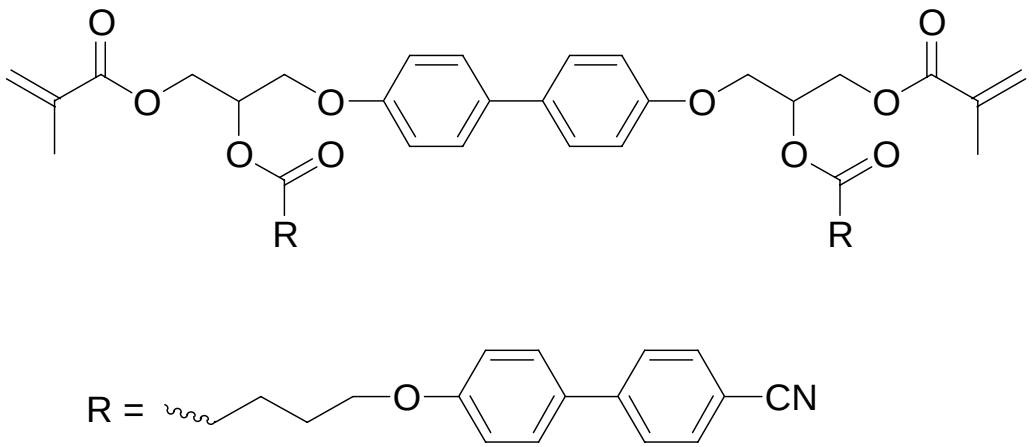

Figure 8. Branched liquid crystalline bismethacrylates.

ORMOCERS

Ormocers (organically modified ceramics), which refers to an inorganic-organic hybrid dental materials is another type of nanostructured hybrid dental materials developed with the purpose of reducing polymerization shrinkage, improving marginal adaption, abrasion resistance and biocompatibility. The ormocers which have an inorganic backbone based on SiO_2 are functionalized with polymerizable organic units such as dimethacrylate and are separated from the trialkoxysilane moiety by different spacer groups (Figure 9). The more rigid the spacer groups, the higher the modulus of elasticity. The nanostructured ormocers can be synthesized from "an alkoxy silane functionalized with a polymerizable group, followed by hydrolysis and condensation which led to an oligomeric Si-O-Si nanostructure" (Figure 10) [68]. However, due to its high viscosity, a diluent, TEGDMA, is often needed. Despite comparable marginal adaptation, a recent study conducted on low shrinkage composites showed that a considerable amount of polymerization shrinkage is still present with this class of materials [11].

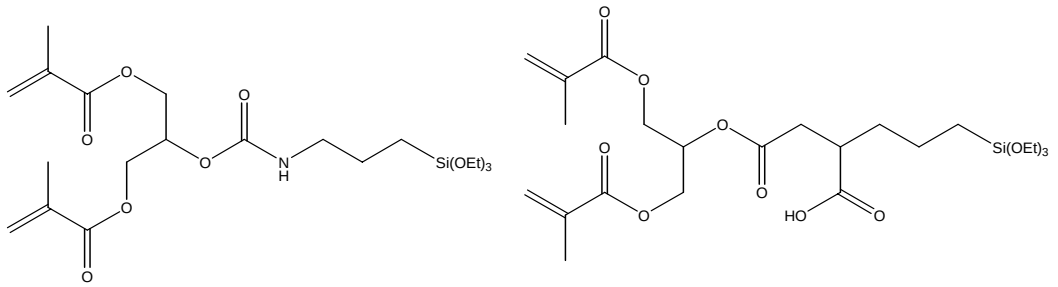

Figure 9. Example of commercially available methacrylate silanes with different spacer group.

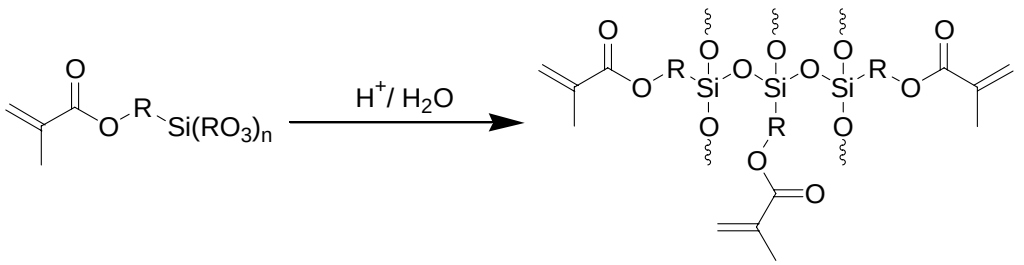

Figure 10. The synthetic route of SiO_2 nanostructures.

SILOXANE

Siloxane dendrimers (Figure 11), which were based on cyclic siloxane cores with photo-polymerizable side groups, were another class of cross-linking monomers developed [69]. This group of material demonstrated increased hardness and reduced viscosity that allowed for high amount of filler loading. As the monomers are designed to link with the siloxane core, uncured monomers linked to the core cannot be easily leached out into surrounding gum tissue. Siloxane dendrimers were found to have useful applications in area of restorative fillings, crowns, bridges or cast restorations.

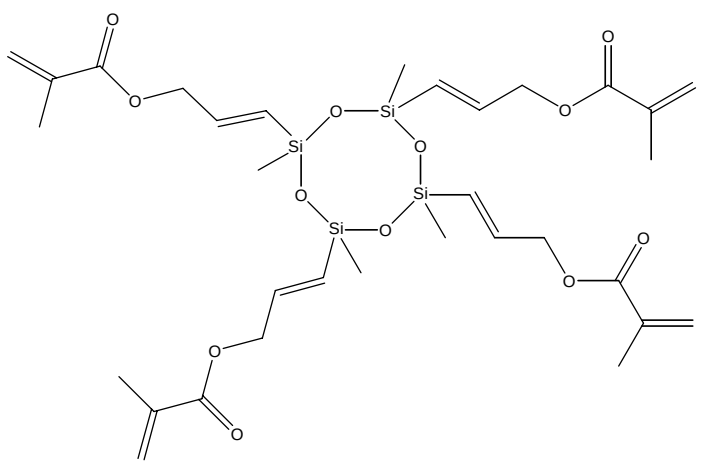

Figure 11. Siloxane dendrimers.

In recent years, 3M ESPE has successfully developed and commercialized the Siloranes™. Siloranes™ (Figure 12), was developed based on the siloxane core and oxiranes. It is a class of cationic ring opening monomers developed to reduce shrinkage. Though initial study based on the Siloranes™ has shown low polymerization shrinkage with comparable elastic modulus and flexural strength, more studies are still warranted [70].

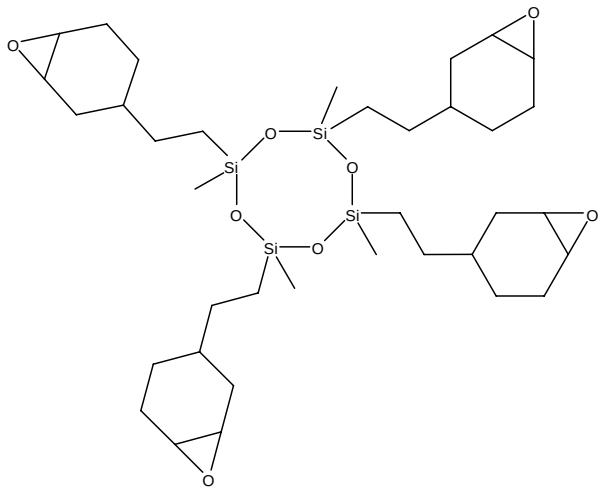

Figure 12. Siloranes™ dendrimers.

POLYHEDRAL OLIGOMERIC SILSESQUIOXANE

Polyhedral Oligomeric Silsesquioxane (POSS™) $(RSiO_{1.5})_x$, with a diameter of 0.54 nm, is one other hybrid organic-inorganic nanocomposite material evaluated for dental applications. POSS™, which can be regarded as the smallest particle of silica, is generally obtained by hydrolysis and condensation of trialkoxy or trichlorosilanes. With a unique well-defined structure, POSS™ is often used for the preparation of hybrid materials with well-defined structures [71]. It can also be chemically functionalized and behave as a platform from which to synthesize organic/inorganic nanocomposites for use in a variety of applications such as performance materials and abrasion resistant coatings.

Incorporation of POSS™ derivatives into polymeric materials can help to improve mechanical properties, increase thermal stability, oxidation resistance and surface hardening as well as to reduce flammability and viscosity during processing. POSS™ monomers, which do not require significant changes in processing, are simply mixed and copolymerized by traditional methods. They form true molecular dispersions when mixed into polymer formulations with no phase separation and hence represent a significant advantage over current filler technologies [72].

In recent years, several POSS™ molecules have been synthesized and investigated for dental applications. Mono-methacrylate functionalized POSS™ (Figure 13) synthesized by Gao *et al.* [73] have been evaluated and used for copolymerization with methacrylate monomers. It was found that incorporation of small amounts (5% *w/w*) of POSS™ molecules resulted in improved mechanical properties and reduced shrinkage. The potential of using

POSS^{TM}-MA (methacryl-POSS^{TM} cage mixture) as a replacement for Bis-GMA has also been investigated [74]. It was also found that a small percentage substitution (mass fraction of 10% or less) of Bis-GMA with POSS^{TM}-MA improved flexural strength and Young's modulus of composites but large percentage substitution (mass fraction of 25% or more) resulted in undesirable mechanical properties, lower degree of conversion and lower reactivity. Liquid epoxy-functionalized cubes (Figure 14) were other POSS^{TM} structures designed for single phase composites with potential application for dental restoratives. This epoxy POSS^{TM} containing up to 65% masked silica was capable of producing hard, scratch- and solvent-resistant materials when photochemically cured [75]. The functionalized POSS^{TM} materials developed thus far for dental applications are all monofunctionalized and work on fully functionalized materials have not been fully explored yet.

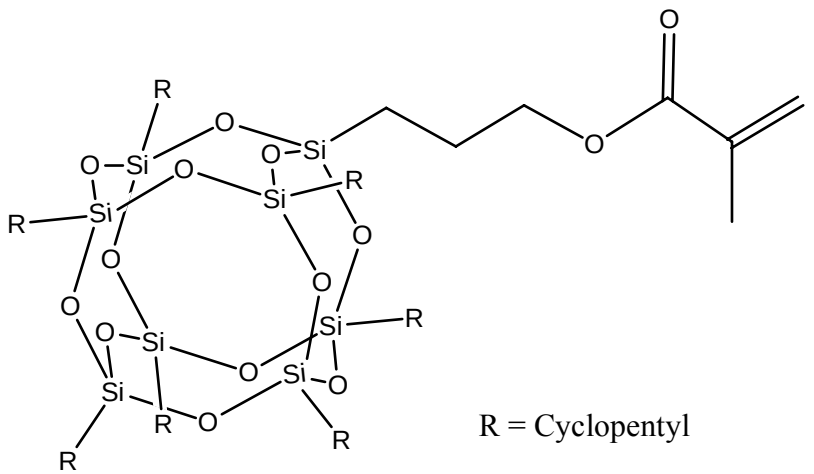

Figure 13. Mono-methacrylate functionalized POSS^{TM}.

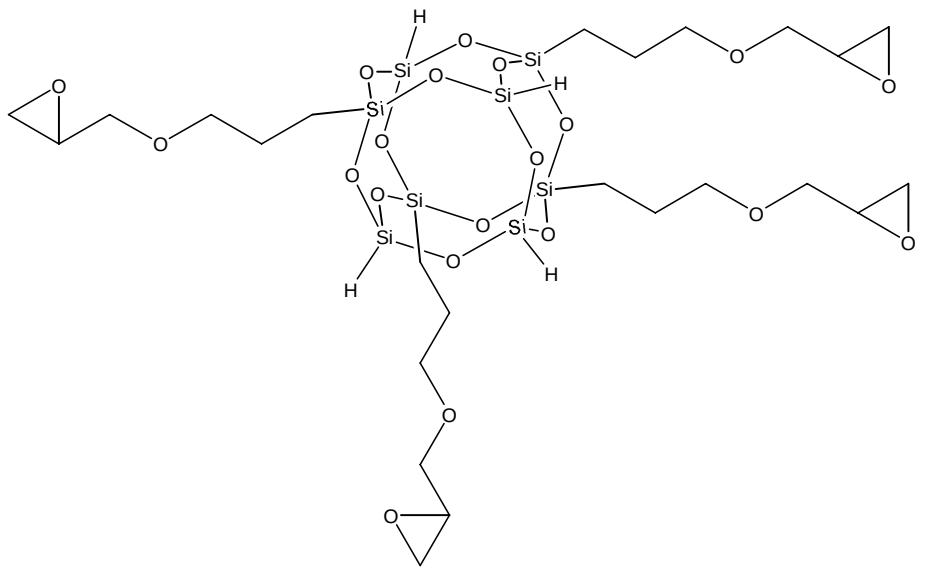

Figure 14. Epoxy functionalized POSS^{TM} structure.

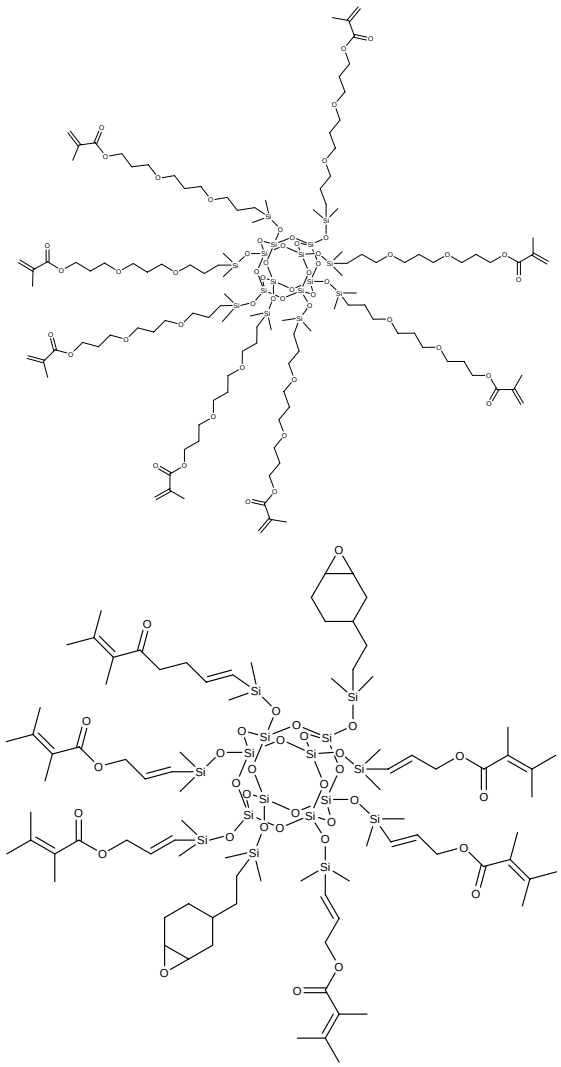

Figure 15. Example of fully functionalized silsesquioxane (SSQ) monomers.

To further evaluate the potential application of POSS^{TM} modified resins as dental monomers, we have designed and developed novel low shrinkage silsesquioxane (SSQ) materials fully functionalized with various methacrylates and epoxy for dental applications (Figure 15) [76-78]. These novel multifunctional SSQ materials were found to have the advantages of being able to be isolated in high yields (>80%) and are viscous liquids that can be polymerized both thermally and photochemically. The SSQ resins were synthesized based on inexpensive starting materials and contained up to 48% masked silica making them ideal for dental materials. The post-gel shrinkage associated with these SSQ compounds were found be significantly lower than the conventional Bis-GMA/TEGDMA mixture. Shrinkage was found to decrease with decrease in the number of methacrylate group attached. In our studies, we have also demonstrated that besides shrinkage, various physico-mechanical properties such as modulus, hardness, stability and cross-link density can also be improved through chain modifications and strategic selection of the polymerizable groups attached to

the SSQ core. The addition of as little as 5 wt% of the SSQ resins into dental monomers helps to reduce shrinkage by as much as 20% [78]. Furthermore, it is anticipated in our study that SSQ compounds will have the advantage of potentially reducing cytotoxic properties as a result of their relatively large size and multi-functionality. For example in current dental composites, because of their small size and limited number of functional groups, uncured monomers can slowly leach out of the composite matrix into surrounding gum/ pulp tissue and cause cytotoxic responses. In the case of SSQs described here, the combined properties of large molecular size (prevents migration) and multiple methacrylate groups (just one reactive site to anchor) makes the probability of leaching out from the matrix extremely low.

CONCLUSION

Research has well documented over the past years that all dental composite shrink during polymerization. While efforts have been made clinically to reduce shrinkage through development of various controlled polymerization techniques, none has been successfully implemented across the whole dental community due to controversy in research findings. While some studies have shown that controlled polymerization techniques help to reduce polymerization shrinkage, stresses and marginal adaption, others have shown otherwise. The use of light-curing techniques such as pulse delay mode was found to result in a more linear polymer structures that is more susceptible to ethanol softening, thus making the technique less attractive for use by clinicians. In order to overcome the problem of polymerization shrinkage, efforts have also been made to develop low/non shrinking materials in the laboratory. While various monomers such as epoxy, spiro-orthocarbonates, ormocers have been developed, none has been successfully implemented commercially. The biggest breakthrough in dental materials research came with the commercialization of the Silorane™ by 3M ESPE. Although shrinkage results are encouraging, more studies are warranted. The development of low shrinking silsesquioxane (SSQ) mutifunctionalized materials developed by our group was also found to be rather encouraging. Thus, the search for dental materials with zero net shrinkage continues to be one of the greatest challenges in composite resins technology.

REFERENCES

[1] Ferracane JL. Current trends in dental composites. *Critical Reviews in Oral Biology and Medicine.* 1995;6:302-18.

[2] Craig RG. Chemistry, composition and properties of composite resins. *Dental Clinics of North America.* 1981;25:219-39.

[3] Dart EC, Nemcek J. Photopolymerisable composition. US Patent 4071424. 1978.

[4] Sturdevant CM, Roberson TM, Heymann HO, Sturdevant JR. The art and science of operative dentistry. St Louis: Mosby. 1995.

[5] Tyas MJ. Color stability of composite resins: a clinical comparison. *Australian Dental Journal.* 1992;37:88-90.

[6] Horsted-Bindslev P. Amalgam toxicity-environmental and occupational hazards. *Journal of Dentistry*. 2004;32:359-65.
[7] Roberts HW, Leonard D, Osborne J. Potential health and environmental issues of mercury-contaminated amalgamators. *Journal of the American Dental Association*. 2001;132:58-64.
[8] Mjor IA. The reasons for replacement and the age of failed restorations in general dental practice. *Acta Odontologica Scandinavica.* 1997;55:58-63.
[9] Mjor IA, Qvist V. Marginal failures of amalgam and composite restorations. *Journal of Dentistry.* 1997;25:25-30.
[10] Soh MS, Yap AU, Siow KS. Post-gel shrinkage with different modes of LED and halogen light curing units. *Operative Dentistry.* 2004;29:317-24.
[11] Yap AU, Soh MS. Post-gel polymerization contraction of "low shrinkage" composite restoratives. *Operative Dentistry.* 2004;29:182-7.
[12] Labella R, Lambrechts P, Van Meerbeek B, Vanherle G. Polymerization shrinkage and elasticity of flowable composites and filled adhesives. *Dental Materials.* 1999;15:128-37.
[13] Burgess JO, Walker R, Davidson JM. Posterior resin-based composite:review of the literature. *Pediatric Dentistry.* 2002;24:465-79.
[14] Letzel H. Survival rates and reasons for failure of posterior composite restorations in multicentre clinical trial. *Journal of Dentistry.* 1989;17:10-7.
[15] Mjor IA, Jokstad A. Five-year study of Class II restorations in permanent teeth using amalgam, glass polyalkenoate (ionomer) cerment and resin-based composite materials. *Journal of Dentistry.* 1993;21:338-43.
[16] Jokstad A, Mjor IA, Qvist V. The age of restorations in situ. *Acta Odontologica Scandinavica.* 1994;52:234-42.
[17] Davidson CL, de Gee AJ. Relaxation of polymerization shrinkage stresses by flow in dental composites. *Journal of Dental Research.* 1984;63:146-48.
[18] Feilzer AJ, de Gee AJ, Davidson CL. Setting stress in composite resin in relation to configuration of the restoration. *Journal of Dental Research*. 1987;66:1636-39.
[19] Sheth JJ, Fuller JL, Jensen ME. Cuspal deformation and fracture resistance of teeth with dentin adhesives and composite. *Journal of Prosthetic Dentistry.* 1988;60:560-69.
[20] Qvist V, Qvist J, Mjör IA. Placement and longevity of tooth-colored restorations in Denmark. *Acta Odontologica Scandinavica.* 1990;48:305-11.
[21] Braga RR, Ferracane JL. Contraction stress related to degree of conversion and reaction kinetics. *Journal of Dental Research*. 2002;81:114-8.
[22] Braga RR, Ballester RY, Ferracane JL. Factors involved in the development of polymerization shrinkage stress in resin-composites: A systematic review. *Dental Materials.* 2005;21:962-70.
[23] Kemp-Scholte CM, Davidson CL. Complete marginal seal of class V resin composite restorations effected by increased flexibility. *Journal of Dental Research.* 1990;69:1243-49.
[24] Choi KK, Cndon JR, Ferracane JL. The effects of adhesive thickness on polymerization contraction stress of composite. *Journal of Dental Research.* 2000;79:812-7.
[25] Uno S, Asmussen E. Marginal adaptation of a restorative resin polymerized at reduced rate. *Scandinavian Journal of Dental Research.* 1991;99:440-4.

[26] Goracci G, Mori G, Casa de'Martinis L. Curing light intensity and marginal leakage of resin composite restorations. *Quintessence International.* 1996;27:355-62.

[27] Koran P, Kürschner R. Effect of sequential *versus* continuous irradiation of a light-cured resin composite on shrinkage, viscosity, adhesion, and degree of polymerization. *American Journal of Dentistry.* 1998;10:17-22.

[28] Lim BS, Ferracane JL, Sakaguchi RL, Condon JR. Reduction of polymerization contraction stress for dental composites by two-step light-activation. *Dental Materials.* 2002;18:436-44.

[29] Friedl KH, Shmalz G, Hiller KA, Markl A. Marginal adaptation of class V restorations with and without "softstart-polymerization". *Operative Dentistry.* 2000;25:26-32.

[30] Bouschlicher MR, Rueggeberg FA, Boyer DB. Effect of stepped light intensity on polymerization force and conversion in a photoactivated composite. *Journal of Esthetic Dentistry.* 2000;12:23-32.

[31] Lopes LG, Franco EB, Pereira JC, Mondelli RFL. Effect of light-curing units and activation mode on polymerization shrinkage and shrinkage stress of composite resins. *Journal of Applied Oral Science.* 2008;16:35-42.

[32] Pfeifer CSC, Braga RR, Ferracane JL. Pulse-delay curing: influence of initial irradiance and delay time on shrinkage stress and microhardness of restorative composites. *Operative Dentistry.* 2006 ;31:610-5.

[33] Price RB, Rizkalla AS, Hall GC. Effect of stepped light exposure on the volumetric polymerization shrinkage and bulk modulus of dental composites and an unfilled resin. *American Journal of Dentistry.* 2000;13:176-80.

[34] Yap AU, Ng SC, Siow KS. Soft-start polymerization: influence on effectiveness of cure and post-gel shrinkage. *Operative Dentistry.* 2001;26:260-6.

[35] Alonso RCB, Correr GM, Cunha LG, De Moraes Souto Pantoja CA, Puppin-Rontani RM, Sinhoreti MAC. Modulated photoactivation methods--effect on marginal and internal gap formation of restorations using different restorative composites. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 2007;82:346-51.

[36] Mehl A, Hickel R, Kunzelmann KH. Physical properties and gap formation of light-cured composites with and without 'softstart-polymerization'. *Journal of Dentistry.* 1997;25:321-30.

[37] Kanca J, 3rd, Suh BI. Pulse activation: reducing resin-based composite contraction stresses at the enamel cavosurface margins. *American Journal of Dentistry.* 1999;12:107-12.

[38] Santos AJ, Lisso MT, Aguiar FH, Franca FM, Lovadino JR. Effect of stepped exposure on quantitative in vitro marginal microleakage. *Journal of Esthetic and Restorative Dentistry.* 2005;17:236-42.

[39] Barros GK, Aguiar FH, Santos AJ, Lovadino JR. Effect of different intensity light curing modes on microleakage of two resin composite restorations. *Operative Dentistry.* 2003;28:642-6.

[40] Hofmann N, Markert T, Hugo B, Klaiber B. Effect of high intensity vs. soft-start halogen irradiation on light-cured resin-based composites. Part I. Temperature rise and polymerization shrinkage. *American Journal of Dentistry.* 2003;16:421-30.

[41] Cunha LG, Alonso RCB, Pfeifer CSC, Correr Sobrinho L, Ferracane JL, Sinhoreti MAC. Modulated photoactivation methods: Influence on contraction stress, degree of

conversion and push-out bond strength of composite restoratives. *Journal of Dentistry*. 2007;35:318-24.

[42] Hofmann N, Denner W, Hugo B, Klaiber B. The influence of plasma arc vs. halogen standard or soft-start irradiation on polymerization shrinkage kinetics of polymer matrix composites. *Journal of Dentistry.* 2003;31:383-93.

[43] Yap AU, Soh MS, Siow KS. Post-gel shrinkage with pulse activation and soft-start polymerization. *Operative Dentistry.* 2002;27:81-7.

[44] Yap AU, Soh MS, Siow KS. Effectiveness of composite cure with pulse activation and soft-start polymerization. *Operative Dentistry.* 2002;27:44-9.

[45] Chye CH, Yap AU, Laim YC, Soh MS. Post-gel polymerization shrinkage associated with different light curing regimens. *Operative Dentistry.* 2005;30:474-80.

[46] Neo BJ, Soh MS, Teo JW, Yap AU. Effectiveness of composite cure associated with different light-curing regimes. *Operative Dentistry.* 2005;30:671-5.

[47] Asmussen E, Peutzfeldt A. Influence of pulse-delay curing on softening of polymer structures. *Journal of Dental Research.* 2001;80:1570-3.

[48] Asmussen E, Peutzfeldt A. Two-step curing: influence on conversion and softening of a dental polymer. *Dental Materials.* 2003;19:466-70.

[49] Soh MS, Yap AU. Influence of curing modes on crosslink density in polymer structures. *Journal of Dentistry*. 2004;32:321-6.

[50] Yap AU, Han VT, Soh MS, Siow KS. Elution of leachable components from composites after LED and halogen light irradiation. *Operative Dentistry.* 2004;29:448-53.

[51] Stansbury JW. Synthesis and evaluation of new oxaspiro monomers for double ring-opening polymerization. *Journal of Dental Research.* 1992;71:1408-12.

[52] Stansbury JW. Ring-opening polymerization of a 2-methylene spiro orthocarbonate bearing a pendant methacrylate group. In: Shalaby SW, Ikada Y, Langer R, Williams J, editors. Polymers of Biological and Biomedical Significance: ACS Symposium series 540; 1993.

[53] Byerley TJ, Eick JD, Chen GP, Chappelow CC, Millich F. Synthesis and polymerization of new expanding dental monomers. *Dental Materials.* 1992;8:345-50.

[54] Eick JD, Byerley TJ, Chappell RP, Chen GR, Bowles CQ, Chappelow CC. Properties of expanding SOC/epoxy copolymers for dental use in dental composites. *Dental Materials.* 1993;9:123-7.

[55] Miyazaki K, Takata T, Endo T, Inanaga A. Thermal and photopolymerization of (meth)acrylates containing a spiro ortho ester moiety and the properties of poly(meth)acrylates. *Dental Material Journal.* 1994;13:9-18.

[56] Tilbrook DA, Clarke RL, Howle NE, Braden M. Photocurable epoxy-polyol matrices for use in dental composites I. *Biomaterials.* 2000;21:1743-53.

[57] Nuyken O, Böhner R, Erdmann C. Oxetane photopolymerization - A system with low volume shrinkage. *Macromolecular Symposia.* 1996;107:125-38.

[58] Sasaki H, Kuriyma A, Kakuchi T. Photoinitiated cationic polymerization of 3-Ethy-3-(phenoxymethyl)-oxetane in nitrogen atmosphere. Journal of Macromolecular Science. Part A: Pure and Applied Chemistry. 1995;32: 1699-1707.

[59] Belfield KD, Abdelrazzaq FB. Photoinitiated cationic cross-linking of 4-methylene-2-phenyl-1,3-dioxolane with 2,2'-(1,4-phenylene)bis(4-methylene-1,3-dioxolane). *Macromolecules.* 1997;30:6985-8.

[60] Moszner N, Völkel T, Zeuner F, Rheinberger V. Radical ring-opening monomers for dental composites. *Polym Preprints.* 1997;38:86-7.

[61] Evans RA, Rizzardo E. Free-radical ring-opening polymerization of cyclic allylic sulfides. *Macromolecules.* 1996;29:6983-9.

[62] Moszner N, Zeuner F, Völkel T, Rheinberger V. Polymerization of cyclic monomers. VII. Synthesis and radical polymerization of 1,3-bis[1- alkoxycarbonyl-2-vinyl cyclopropane-1-yl)carboxyl] benzenes. *Journal of Applied Polymer Science* 1999;72:1775-82.

[63] Moszner N, Zeuner F, Rheinberger V. Polymerization of cyclic monomers. 4. Synthesis and radical polymerization of bi-and trifunctional 2- vinylcyclopropanes. *Macromolecular Rapid Communications.* 1997;18:775-80.

[64] Rawls HR, Wellinghoff VT, Norling BK, Leamo SH, Swynnerton NF, Wellinghoff ST. Low shrinkage resins from liquid crystal diacrylate monomers. *Polymer Preprints.* 1997;38:167-8.

[65] Ritter H, Draheim G, Moszner N, Salz U. Dental materials based on liquid crystalline monomers. US Patent 6031015. 2000.

[66] Hölter D, Frey H, Mülhaupt R, Klee JE. Ambient-temperature liquid crystalline bismethacrylates based on cholesterol: cholestric and smectic thermosets. *Advanced Material.* 1998;10:864-8.

[67] Hölter D, Frey H, Mülhaupt R, Klee JE. Liquid crystalline thermosets based on branched bismethacrylates. *Macromolecules.* 1996;29:7003-11.

[68] Wolter H, Glaubitt W, Rose K. Multifuctional (meth)acrylate alkoxysilanes. A new type of reactive compounds. *Materials Research Society Symposia Proceedings.* 1992;271:719-24.

[69] Bissinger P, Gasser O, Guggenberger R, Soglowek W, Eckhardt G, inventors; Cyclosiloxane-based cross-linker monomers, production thereof and use in polymerizable materials. WO 00/42092, ESPE Dental AG.2000.

[70] Weinmann W, Thalacker C, Guggenberger R. Siloranes in dental composites. *Dental Material.* 2005;21:68-74.

[71] Li G, Wang L, Ni H, Pittman JCU. Polyhedral oligomeric silsesquioxane (POSS) polymers and copolymers: A review. *Journal of Inorganic and Organometallic Polymer.* 2001;11:123-54.

[72] Haddad TS, Stapleton R, Jeon HG, Mather PT, Lichtenhan JD, Phillips S. Nanostructured hybrid organic/inorganic materials. Silsesquioxane modified plastics. *Polymer preprints.* 1999;40:496.

[73] Gao F, Tong Y, Schricker SR, Culbertson BM. Evaluation of neat resins based on methacrylates modified with methacryl-POSS, as potential organic-inorganic hybrids for formulating dental restoratives. *Polymers for Advanced Technologies.* 2001;12:355-60.

[74] Fong H, Dickens SH, Flaim GM. Evaluation of dental restorative composites containing polyhedral oligomeric silsesquioxane methacrylate. *Dental Materials.* 2005;21:520-9.

[75] Sellinger A, Laine RM. Silsesquioxanes as synthetic platforms. 3. Photocurable, liquid epoxides as inorganic/organic hybrid precursors. *Chemistry of Materials.* 1996;8:1592-3.

[76] Soh MS, Yap A, Sellinger A. Methacrylate and epoxy functionalized nanocomposites based on silsesquioxane cores for use in dental applications. *European Polymer Journal.* 2007;43:315-27.

[77] Soh MS, Yap AU, Sellinger A. Physicomechanical evaluation of low-shrinkage dental nanocomposites based on silsesquioxane cores. *European Journal of Oral Sciences.* 2007;115:230-8.

[78] Soh MS, Yap AU, Sellinger A. Effect of chain modifications on the physicomechanical properties of silsesquioxane-based dental nanocomposites. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 2008;85:78-86.

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Chapter 3

SIMULTANEOUS FLUORESCENCE AND REFLECTION CONFOCAL MICROSCOPY STUDY OF LIVING OSTEOBLAST BIOENERGETICS AS A TOOL FOR THE DESIGN OF SURFACE TOPOGRAPHY OF DENTAL IMPLANTS

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ABSTRACT

Confocal laser scanning microscope (CLSM) appears to be a valuable and non invasive tool for simultaneous 3D visualization of both cell morphology and structure and material scaffolds. The CLSM is also capable of creating excellent images using backscattered or reflected light. Confocal backscattered imaging is particularly useful for creating 3D images of the surface of materials and profilometric studies. For the quantification of maximal and minimal values in surface profile, distance between peaks in each surface, width of grooves and valleys, and axial ratio of the cells growing on the different surfaces were assessed. In addition to image data of cell morphology, microstructural data of scaffolds and cell-scaffold interaction can be readily obtained using this method. The quality of cell adhesion to the dental implant surface determines the tissue integration, and the surface can directly influence osteoblast adherence, attachment, spreading and metabolism, modifying and controlling the osseointegration process. The combinated use of reflectance and fluorescence modes is of capital interest to provide detailed images of living cell interactions with underlying surfaces in biomedical devices as dental implants, building the cellular and molecular basis of mechanotransduction, a process where high amounts of energy are demanded. Consistently with this, mitochondria that accumulate at sites of development of focal

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adhesions, mechanosensory devices, exhibit higher membrane potential compared with mitochondria that are not at those sites.

The methodology described herein is useful to directly visualize and quantify the role of underlying environmental cues for force-generating and anchoraging activities on mitochondrial bioenergetics in human living osteoblasts, growing on a number of customized biomaterials surfaces, and permit the use as a tool for designing the surface quality in order to provide the ideal surface for osseointegration of dental implants.

1. INTRODUCTION

The ageing of the population and the greater expectation towards the quality of life have resulted in an increasing global demand for orthopaedic implants to replace or repair damaged bones and joints. Titanium is particularly used as a component of articular prosthesis since it is a biocompatible materials with a relatively high mechanical constraint, bioinert property and a corrosion resistant surface oxide layer [Rouahi et al., 2006].

It is now well accepted that the complex three-dimensional structural-mechanical environment of cells in vivo, including time-varying changes in stresses and strains, significantly influences the fundamental cellular response in terms of cell morphology, phenotype, and function. It is likely, therefore, that the ability of an engineered biomaterial or tissue construct to approximate the structural–mechanical aspects of the cellular microenvironment is an important factor in determining the eventual success or failure of such engineered devices when used clinically for tissue repair or replacement. Recently, a so called "self-induced mechanotransduction" mechanism has been suggested, based on the hypothesis that surface topography alters nuclear morphology and chromosome position in adherent cells, leading to changes in gene transcription [Lim et al., 2007, Dalby 2005] Understanding how the cellular response proceeds within the three-dimensional context of structural and mechanical restraints offered by the extracellular matrix will drive the next generation of engineered scaffolds and devices to be used for repair and replacement of defective tissues. [Voytik-Harbin et al., 2003].

1.1. Osseointegration

Among the capital rules for the design and selection of the core biomaterials we must consider the real condition of the damaged tissue as well as the degree of functional alterations, as result of the damage, that should be restored. We can define a biomaterial as any material that is tolerated by the organism and can, thus, have a protesic use. In the case of dental implants, their success is measured by the degree of osseointegration. We can define osseointegration as "a direct functional and structural relationship between the living bone and the implant surface [Branemark, et al., 1999]. In consequence, a detailed knowledge of bone tissue behavior is needed for the successful development of any biomaterial designed to be used in implanthology.

The osseointegration concept has evolved as new research techniques in tissue engineering have been developed. For some authors, the term *"osseointegration" should be substituted for the term " biointegration", a process by which a rigid fixation, clinically*

asyntomatic is achieved, with alloplastic material,that remains in bone during functional charges " [Albrektsson & Wennerberg, 2005].

Clasically, the limiting factors for osseointegration to be achieved in success, as described by Albrektsson, can be cited as follows: biocompatibility, design, implant surface, quality of receptor´s bone, surgical technique, and prosthesis (in reference to the charge that bone will hold). Furthermore, dental implants must be resistant to bacterial colonization, the inflammatory response should be reduced to minimum, and soft tissue overgrowth and collapse to bone tissue formation needs to be avoided.

At present, the traditional protocol of dental implants has changed, it has been simplified and the implant-supported restorations shows predictable long term results. The term immediate-loading is used to designate dental implants subjected to occlusal functional loading immediately after implant placement [Gallucci, 2007] without time for full osseointegration. Three predominant biologic factors emerge in consideration of osseointegration and immediate loading: (1) factors affecting interfacial bone formation (osteogenesis); (2) micromotion effects on periimplant osteogenesis; and (3) periimplant bone resorption (osteolysis). [Cícero & Daut 2003]. The most important aspect of early periimplant healing is the recruitment of osteogenic cells and their migration to the implant surface [Davies, 2003], avoiding the possible citotoxic or osteolytic response and, therefore, the presence of connective tissue.

In order to design better implant materials it is important to understand the events at the bone-material interface. At present, it is widely accepted that the surface properties play a critical role in long-term stability and functionality of the implant. The material surface must be able to recruit bone-forming cells, i.e., osteoblasts, such that they can colonize and synthesize new bone. It is well established that surface roughness plays an important role in implant fixation and can directly influence osteoblasts adherence, attachment, spreading and metabolism modifying and controlling the osseointegration process. [Degasne et al. 1999; Ivanoff et al. 2001; Keller et al. 2003; Linez-Bataillon et al. 2002; Papalexiou et al. 2004; Sader et al. 2005, Popat et al., 2007]

2. CONFOCAL LASER SCANNING MICROSCOPY IN DENTAL MATERIAL RESEARCH

2.1. Confocal Principles

The principle of the confocal laser scanning microscopy (CLSM) is the capability of removing from the image, by the use of an adequately positioned "pinhole", the out of focus light. This allows one the acquisition of images with exceptional resolution, and also the collection of optical slices of the specimens. But, in addition, all these exceptional images can be collected from intact specimens, even non transparent ones, as is the case for the titanium implants that we describe in the chapter, and used to create 3D reconstructions of the sample.

This capability successfully enhance the possibility of collecting information in the real, non manipulated specimens, that in the case of surface characterization appears to be of singular interest. The combined use of immunolabelling of those cell features of interest

(morphological, functional or both) opens an interesting field for the intensive analysis of cell-surface interactions.

As we describe in this chapter, not only the fluorescence mode of CLSM offers exceptional resolution, but also the backscattered mode. The combined use of both applications appears to be a valuable and non invasive tool for simultaneous visualization and 3D reconstruction of both cell morphology and structure, and material scaffolds [Tan et al., 2004a, Tan et al. 2007, Vilches et al.,2007], together with the identification of fluorescent functional markers applied to living cells.

The confocal laser scanning Leica TCS SL inverted microscope employed in the study uses spectral separation of various wavelengths of fluorescent light as a valuable alternative method to the most traditional use of individual dichroic mirrors and optical filters. The microscope uses a prism to separate the light into its component colours, which are then directed to individual detection channels. Although this system highly increases the complexity in the use of the microscope, one of the most important advantages of spectral separation is the greatly increased versatility of the instrument in both defining spectral regions that are directed to individual channels and the ability to separate fluorophores with highly overlapping emission spectra [Hibbs, 2004].

The quality of laser laser light that will reach the specimen is princeps for confocal image acquisition. Not only the proper laser alignment, but also the full control of the intensity of laser light is of capital interest. For all the experiences described in this chapter, the laser intensity at the sample was kept to the lowest power able to produce a fluorescent signal, and maximum voltage of photomultipliers was used to decrease the required laser power as much as possible. All samples were exposed to laser for a time interval not higher than 5 min, not only to avoid photobleaching of immunolabelled actin filaments, but also to minimize the reflection from the titanium surfaces. A pinhole size of 1 Airy disk was used in this study for image acquisition both for surface topographical features obtained with the backscattered mode and for the assessment of cellular behavior in response to the structural cues from the scaffold, with the fluorescence mode.

In order to achieve the most favourable conditions for the non-invasive analysis of osteoblasts-implants interface, including cytoskeletal organization, cell adhesion and mitochondrial bioenergetics, as indicators of implant suitability, special care was dedicated to keep the optimal working distance between lens and specimen. In confocal microscopy the objective also becomes the condenser of laser light, and thus, needs to combine a high degree of optical correction with good throughput and a minimum of internal stray light or photon noise generation [Keller, 2005]. For these reasons, image acquisition was carried out with a glycerol/water immersion objective, with a long working distance and adjustable collar for correcting for coverslip thickness. In a confocal microscope, the WD sets an absolute limit that the focus plane can be below the top surface of the coverslip. This is particularly relevant for combination studies with fluorescence and backscattered images, as the absorption properties of the scaffold (i.e., opacity) can have a negative impact on image contrast in the backscattered (BSL) mode [Viches et al. 2007]. The adequate correction for 0.17 mm thickness coverslips, achieved in this study by manipulation of the adjustable collar of the 63.0 x 1.30 glycerol objective, is of paramount importance for image acquisition. The spherical aberration induced by non-specified coverslip thickness leads to loss of energy at the pinhole, reduced depth discrimination, and an axial shift of the best focus [Keller, 2003, Vilches et al., 2007]. For the visual control of adjustment, the flat

surface of the coverslip should be imaged as a thin horizontal line when scanning the sample in the xz mode (Figure 1).

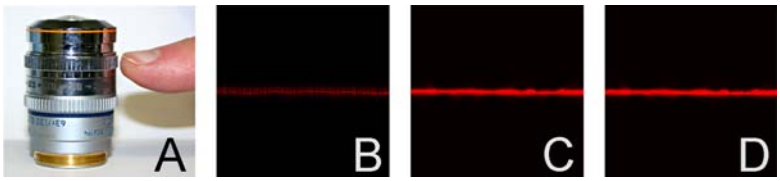

Figure 1. A. Adjustable collar of the 63x1.30 glycerol/water immersion objective. (B.C.D. Sequential image acquisition, in the xz scanning mode during the adjustment process for coverslip thickness. Optimal correction is achieved when a sharp interface image is obtained, as shown in D.

2.2. Backscattered Light Imaging

Reflected light confocal imaging is more properly known as backscattered light imaging to emphasize the point that the light been collected is that which is "scattered" by the sample, rather than the direct reflection from the sample that, in fact, often seriously degrades the quality of the image. In this approach the primary dichroic mirror is replaced with a neutral beam splitter that transmits the laser light scattered by the specimen back to the detector. [Hibbs, 2004, Vilches et al., 2007]

3. Confocal Backscattered Mode for Dental Implants Surface Characterization

3.1. Dental Implants Surface Characterization

In order to select the appropiate surface for osteoblast attachment, a detailed knowledge of biomaterial topography is quite helpful.

The cells and disks were simultaneously visualized using a Leica TCS-SL confocal microscope equipped with a 63.0x1.30 glycerol objective. Images were collected and processed for the quantitative analysis using the imaging software provided by the Leica TCS SL system. Profilometric studies, for the quantification of maximal and minimal values in surface profile, distance between peaks in each surface, width of grooves and valleys, and axial ratio of the cells growing on the different surfaces were assessed. All samples were exposed to laser for a time interval not higher than 5 min to avoid photobleaching. The excitation beam splitter selected was a DD 488/543. The laser was set to the lowest power that was able to produce a fluorescent signal. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible, the lowest voltage being 279.3 V and the highest voltage 778.7 V. Offset was maintained at 0. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 512x512, with a mean voxel size of 209.20 nm. Series were acquired in the xyz mode.

As shown in the figures below, (Figure 2) the methodology employed and the use of CLSM allows surface characterization in detail. Not only morphological information

(presence of peaks, caves, grooves or valleys) can be retrieved from the specimen, but also profilometric measurements along selected lines and/or planes of interest can be quantified. Thus, height of peaks, width, diameter or depth of any topographical feature of interest can be measured. The topographical 3D reconstruction of series is quite helpful for the proper understanding of the micro or nanotopographies tested. This appears to be quite evident in some of the images presented (Figure 2). Combined 3D reconstruction also provides interesting information about mechanotransduction-induced phenotypical changes in osteoblast growing on the different surfaces tested.

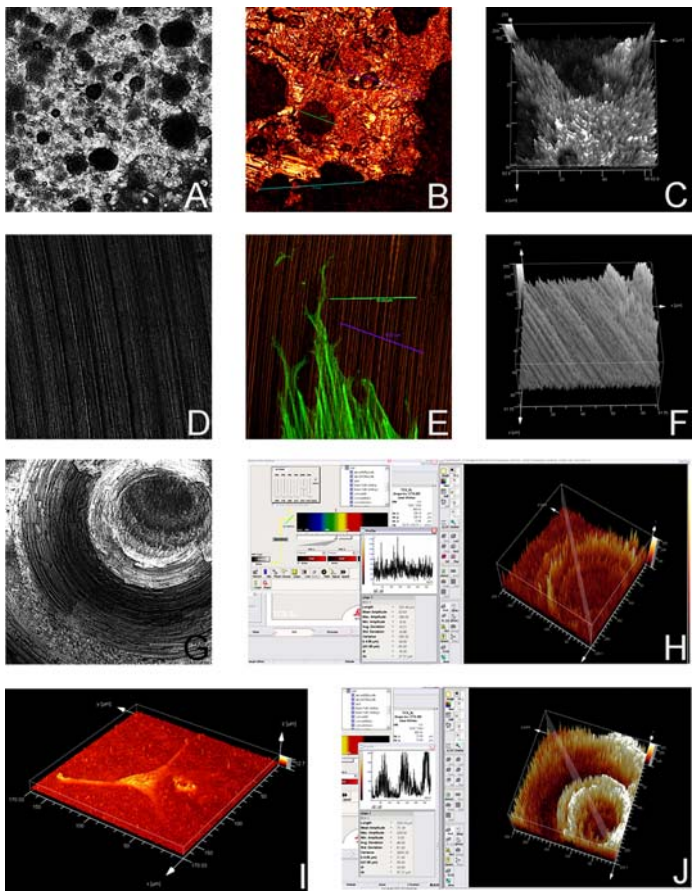

Figure 2. CLSM (backscattered mode) of the different surfaces tested. A: Rough surface, with prominent peaks and caves. Image acquired in the xyz mode. Magnification 63x, zoom 1.2. B. When a glow palette is used as pseudocolor, the most prominent zones appear in yellow. Magnification 63x, zoom 1.8 As shown, quantification of surface features (height, depth..) can be carried out. The topographical 3D reconstruction, in C. is a valuable tool for surface characterization. D. Machined surface (backscattered mode), magnification 63x, zoom 1.2. E. Combined backscattered and fluorescence image obtained from a rhodamine-phalloidin stained osteoblast growing on the machined surface. Actin fibers (green) are mostly running in parallel throughout the grooves and valleys (disk surface, in red) magnification 63x, zoom 1.2. F. Topographical 3D reconstruction of the machined surface.G. Micropatterned surface. 3D reconstruction of a 40 stack series acquired in the xyz scanning mode in CLSM backscattered mode, in which profilometry along a selected line (H) or plane (I) can be performed, magnification 63x, zoom 2. J. Tridimensional topographical reconstruction of cell surface interaction in the rough surface.

4. Combined Fluorescence and Backscattered CLSM for the Asessment of Osteoblast Adhesion to Dental Implants

4.1. Cell Adhesion

The primary host response after implantation is an inflammatory reaction, elicited by the surgical trauma and modified by the presence of the implant. The deposition from osteogenic cells on the implant surface of a layer of non-collagenous proteins that regulate cell adhesion and binding of minerals has been described during the early stages of host response [Kikuchi et al 2005]. The surface characteristics determine how biological molecules will adsorb to the surface, and also the cell behaviour on contact zone. When cells get in contact with the surface, they will firstly attach, then adhere and spread. This first phase depends on adhesion proteins. Thereafter, the quality of this adhesion will influence their morphology,and their capacity for proliferation and differentiation (Davies 2003, Park et al 2005, Anselme 2000).

If cells have not adhered adequately, because of an unstable implant or the chemical and physical properties of the implant surface itself, a fibrous capsule (fibrous encapsulation), with a liquidfilled void, may form between the soft tissue and implant. This may result in the destabilization of the implant, inhibition of tissue regeneration and repair, and an increased chance of infection, because of poor vascularization around the implant and fibrous tissue [Harris et al. 2005]. The interaction of extracellular matrix molecules with the cell generate signals which are transmitted via the integrins to the cytoplasm, cytoskeleton and finally to the nucleus [Linez-Bataillon et al. 2002; Shah et al. 1999b]. Physico-chemical interactions between cells and materials involving ionic forces, van der Waals forces, and other forces govern this attachment process. [Meyer et al. 2005].

Cells adhere to the extracellular matrix (ECM) via integrin-mediated adhesions that link the ECM to the actin cytoskeleton. (Figure 3) Receptors for ECM proteins, transmembrane integrin molecules, are associated via their cytoplasmic domains with a complex of proteins including vinculin, talin, paxillin, tensin, and many others which are all involved in the dynamic association with actin filaments. In cultured cells, integrin based molecular complexes form discrete morphological entities of several types. Small (0.5–1μm) dot-like or point contacts also known as focal complexes are localized at the edges of lamellipodia. Elongated (3–10μm in length) streak-like structures associated with actin- and myosin-containing filament bundles (stress fibers) are known as focal contacts or focal adhesions. An additional form of adhesion site, tensin-enriched fibrillar adhesions, is involved in the fibronectin. In addition to their function as adhesion sites, matrix adhesions participate in adhesion-dependent signaling [Riveline et al., 2001; Zimerman et al. 2004a, Zimerman et al., 2004b, Diener et al., 2005, Schwarz & Bischofs, 2005].

The clinical success of dental implants is closely associated with the properties of implant materials, which affect cell attachment as well as subsequent cell differentiation, matrix production, and mineralization [Okumura et al., 2001].

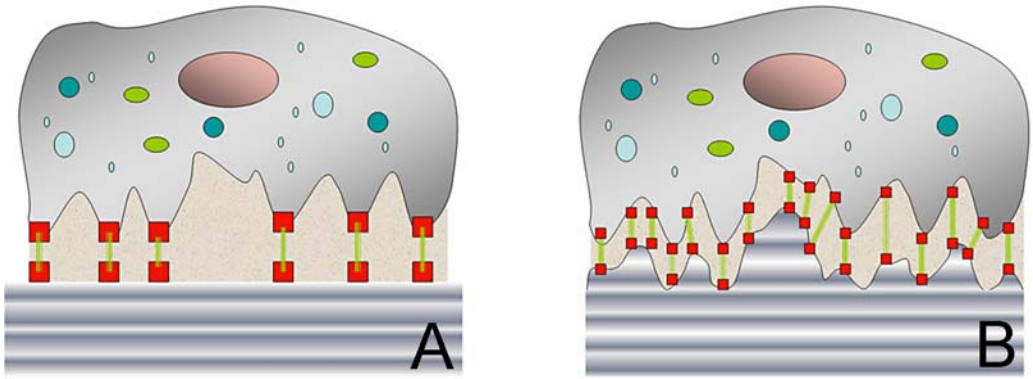

Figure 3. Schematic representation of focal adhesion distribution in machined (A) and rough (B) surfaces.

The adhesion phase occurs over long periods of time and involves various biological molecules (extracellular matrix proteins, cell membrane proteins, and cytoskeleton proteins) which interact together to induce the subsequent cell response in terms of migration and differentiation. The pH as well as the ionic composition and strength of solution, temperature and the functional group of proteins and substrates are the factors determining protein adsorption. Small rapidly diffusing proteins attach early after substrate/protein interaction at the surfaces, but are then replaced by larger proteins with high affinity to the surface. [Meyer et al. 2005].

Initially, the defect between bone and implant is filled with a fibrin network, derived from plasma, which leaks from a damaged blood vessel at the defect´s edge. Then, granulation cells are present in the wound. These cells send out cytoplasmic projections while still moving in the fibrin network. In the next phase, granulation cells stop moving in the wound and their projections connect one to each other to form a cellular network, which is still perfused with erythrocytes from blood vessels. Blood vessels progressively will reduce their number and diameter, creating a characteristic capillary network for the connective tissue. Into this capillary network, fibroblasts from the periosteum, endosteum and red bone marrow invade and produce a network of collagen and osteogenic cells into the network start to produce a callus. Osteogenic cells then develop into osteoblasts, which begin to produce spongy bone trabeculae and is referred to as a bony callus. In the final phase, spongy bone is gradually replaced by compact bone around the periphery.

4.2. CLSM of Osteoblasts Adhesion to Implant Surface

The cells and disks were simultaneously visualized using a Leica TCS-SL confocal microscope equipped with a 63.0x1.30 glycerol objective phalloidin staining of actin cytoskeleton (excitation 554 nm/emission 573 nm) and surface reflectance. (Figure 4). Images were collected and processed for the quantitative analysis using the imaging software provided by the Leica TCS SL system. Profilometric studies, for the quantification of maximal and minimal values in surface profile, distance between peaks in each surface, width of grooves and valleys, and axial ratio ofthe cells growing on the different surfaces were assessed. All samples were exposed to laser for a time interval not higher than 5 min to avoid

photobleaching. The excitation beam splitter selected was a DD 488/543. The laser was set to the lowest power that was able to produce a fluorescent signal. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible, the lowest voltage being 279.3 V and the highest voltage 778.7 V. Offset was maintained at 0. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 512x512, with a mean voxel size of 209.20 nm. Series were acquired in the xyz mode.

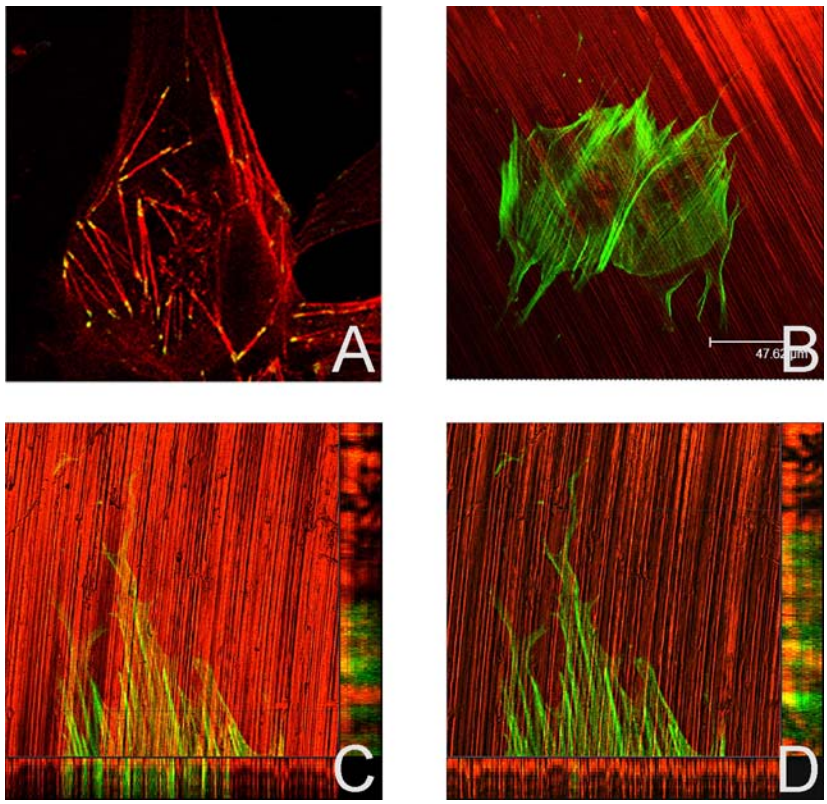

Figure 4. Combined fluorescence labelling of actin stress fibers (rhodamine-phalloidin, in red) and focal contacts (antivinculin antibody, yellow) in a human NHOst cell. Magnification 63x, zoom 1.2. B. Combined backscattered and fluorescence CLSM of human osteoblasts growing on a machined Ti surface. Magnification 63x, zoom 1. C,D. Selected images from a 40 stacks series were a line scan was performed to evaluate cell surface contacts. Both in B,C,D, Green: actin cytoskeleton, red: disk surface. Magnification 63x, zoom 1.5.

4.3. CLSM of Living Osteoblasts

One of the most amazing capabilities of the CLSM in our laboratory is the possibility of carrying out experiences with living cells. In the field of interest of the present chapter, we now describe the main possibilities of the CLSM for the quality control of implant devices, by monitorization of living osteoblasts behavior in the presence of the different biomaterials tested (Figure 5).

Some important requirements must be taken into account when living cells need to be analyzed. The most relevant is, obviously, that cells should be kept not only alive during the experience, but also in non-stress conditions that could disturb any of the cellular functions we are seeking for. In order to ensure cell health, the CLSM is fully covered with an incubation system, that resemble a cell culture incubator. Cells are, then, kept under controlled temperature, 37ºC, pH, 5% CO2, and humidity, by means of a controlled gas-air atmosphere with a 4 l/h flow. (Figure 6)

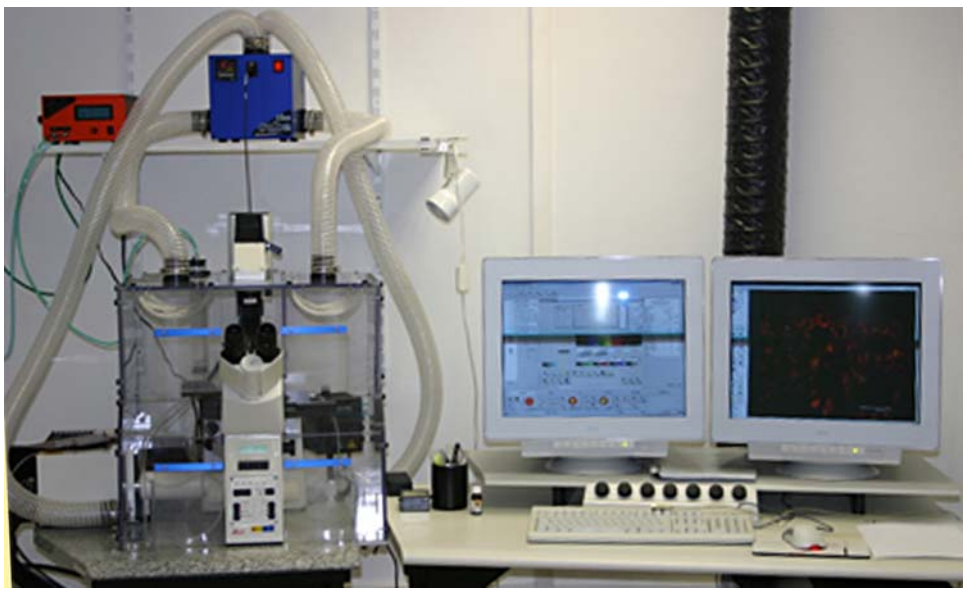

Figure 5. Leica TCS SL confocal laser scanner microscopy in our laboratories. The system is equipped with an incubation system for living cells/ tissues analysis, under controlled temperature and CO2 controlled atmosphere. The system allows fluorescence, backscattered and DIC (Nomarski) analysis of samples.

Figure 6. A.Glass-bottom culture plates employed for CLSM examination of cultured osteoblasts. Plates are ensured into a plate-adapter (B) prior to allocation on the microscope stage. The system includes an adaptor device that suits the plate-adapter and ensures the proper gas-air mixture in the cells environment (C,D,E), as shown in detail. Finally the whole system is carefully placed onto the stage (F,G) and suited to CLSM head prior to scanning.

Image acquisition can be carried out in different CLSM modes, depending on our requirements: DIC (Nomarski mode), fluorescence or backscattered modes, all alone or in combination, and single images or series of stacks can be obtained. Finally, we can examinate the cells for a short period of time or, if necessary, we can develop time-lapse studies along svereal hours or days. An exquisite care should be observed concerning cell exposition to laser, that should be reduced to the minimum required to obtain an image. Heath excess and photobleaching can be induced to cultured osteoblasts, with alteration of the determinations obtained, if the procedure is not accurate.

4.4. Time-Lapse Studies

With the term "time lapse" we make reference to an experimental design that can obtain data from living cells along time. In this chapter, some images obtained at different culture times from cells kept alive inside the incubation system are presented (Figure 7).

When analyzing osteoblast response to micro or nanocues from implant devices, the information obtained in vivo concerning morphological changes or biochemical reponses is of extraordinary interest. As shown, high resolution images of cellular movement, filopodial and lamellopodial protrusions or even cell division can be obtained.

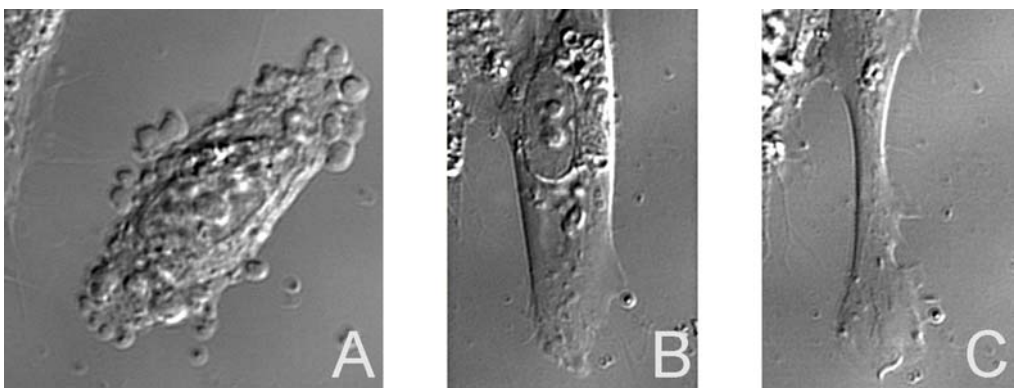

Figure 7. Living NHOst cells during time-lapse studies in Nomarski mode. In A. an isolated osteoblast starts to adapt and move. B and C. subsequent adaptative changes, i.e. lamellopodial development and cell sharpening can be observed. Magnification 63x, zoom 1.

Technically, great care should be taken when programming the time-lapse recording. The system is capable to obtain an image (in the xyt or xyzt scan modes) at the programmed time interval, ranging from seconds to some minutes or days during a previously established time period. It is necessary to remark that, not only if fluorescent probes are to be used, a detailed knowledge of cellular physiology is also required.

Two of the most relevant applications of the living cell study with the CLSM in dental material research regarding cell-implant microenvironment will now be described: the role of surface in promoting the essential initial phenomena for osteoblasts adhesion to the surface tested and the assessment of mitochondrial bioenergetics as indicator of the quality of osteoblasts adhesion, that will, subsequently, improve new bone formation.

4.5. Osteoblast Migration and Adhesion

The process of cell adhesion on materials depends on various parameters: the topography, the chemistry, or the composition of the material. The term 'adhesion' in the biomaterial domain covers different phenomena. It is a step-by-step process from the initial contact to a long-term cell response. The whole interaction can be divided in different events, which are acellular at first time and cellular in second time. Protein adsorption is well known to be the first event that takes place after contact with body fluids and is influenced by physicochemical characteristics of the materials. It is followed by the attachment phase which occurs rapidly. Over a longer period, strong adhesions are formed with the involvement of various biological molecules: cytoskeleton proteins, cell membrane proteins and extracellular matrix proteins which interact together for inducing signal transduction, promoting the action of transcription factors and consequently regulating gene expression. Hence, the adhesion phase controls the subsequent cell response in terms of migration and differentiation [Rouahi et al., 2006].

The adhesion model would include at least three consequent steps:1) Recognition and establishment of contacts between pericellular components and complementary binding domains 2) Pericellular component-mediated contacts trigger the approach of the membrane-bound receptors to the substrate. 3) Interactions between integrins in the membrane and their binding domains on the surface initiate focal contact development and maturation, including linking to the cytoskeleton [Cohen et al., 2004]

During cell spreading and locomotion the assembly of early cell contacts to the ECM at the leading edge are driven by actin polymerization. In the nearby lamella, actomyosin contraction plays a major role in regulating FA structure and dynamics as well as the position of the cell's front consequently affecting the progression of the spreading or migration process. These protrusive and retractive events are tightly regulated by the small GTPases Rac1 and Rho,which trigger actin polymerization in the leading edge and actomyosin contractility, respectively. Local tension generated by actomyosin contractility and transduced via integrin adhesions, can affect these processes by modulating FA dynamics, membrane protrusion, and tail retraction. Thus, the focal complexes and FA that are formed during spreading serve as cytoskeletal organizing centers as well as surface-sensing entities that control, locally and globally, adhesion-mediated signaling and coordinate the adhesive and migratory process. [Cavalcanti-Adam et al., 2007].

Cell migration requires a dynamic interaction between cell, substrate and cytoskeleton. First, cells develop a protrusion of their leading edge to form a lamellipodium. Second, after lamellipodium formation and fixation, cells use adhesive interactions to generate the traction and energy required for cell movement. The last step of the migratory cycle is the release of adhesions at the rear part of the cell followed by its detachment and retraction (Figure 8). Integrins have been implicated in cell migration. In general, cells with a low motility form strong focal adhesions while motile cells form less adhesive structures. An intermediate level of attachment force induces a maximal migration rate [Karp 2007].

Filopodia are active extensions of membrane and cytoskeleton beyond the cell edge into previously unoccupied areas; therefore, these structures are frequently associated with the exploratory behavior of the leading edge of a motile cell. Retraction fibers are due to retraction and condensation of membrane and cytoplasm around a residual cytoskeletal core. In migrating cells they represent the trailing edge and are thought to provide a specialized

mechanism by which the trailing edge is released from the substratum to allow migration in the opposite direction (Figure 9). These trailing structures are thought to be less structurally rigid and more deformable than lamellipodial and filopodial structures at the leading edge of the cell [Cavalcanti-Adam et al., 2007, Lodish et al. 2005].

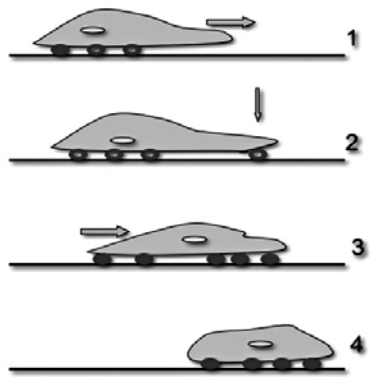

Figure 8. Cell movement, schematical original representation: 1: protrusion, B: loosening, 3: translocation, 4: adhesion.

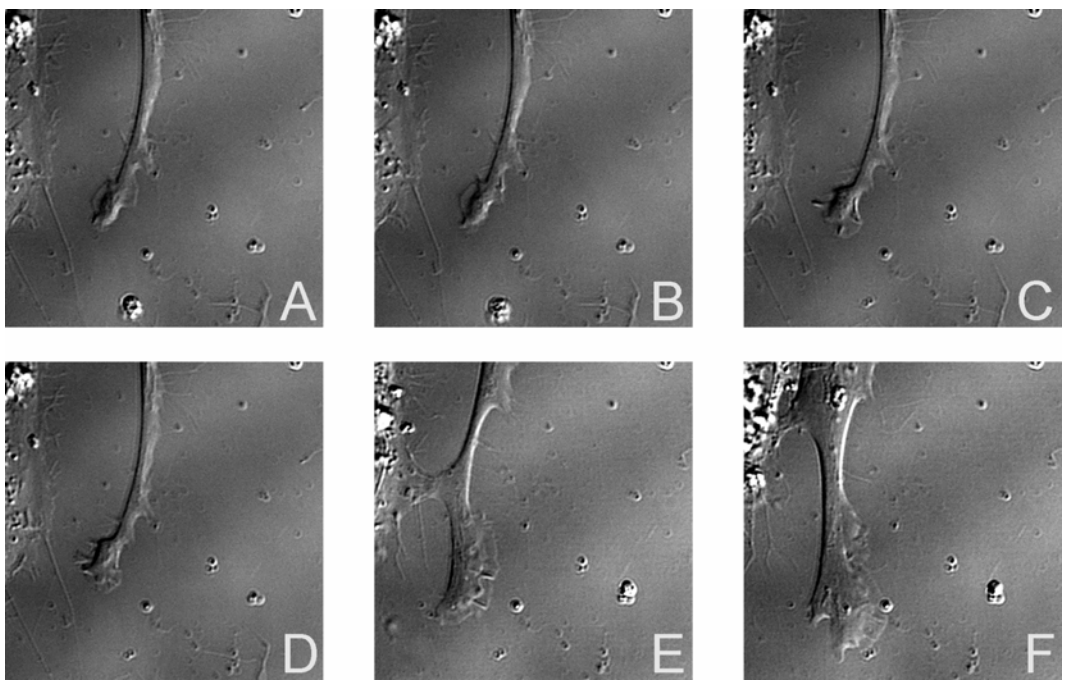

Figure 9. Time lapse acquisition (Nomarski mode). Selected images from a 48h experience on living osteoblasts, that show the sequential cell approach to surface and differentiation with filopodial and lamellopodial emission. Magnification 63x, zoom 1.

5. COMBINED FLUORESCENCE AND BACKSCATTERED MODES FOR THE STUDY OF MITOCHONDRIAL BIOENERGETICS IN LIVING OSTEOBLASTS GROWN ON DENTAL IMPLANTS

5.1. Mechanotransduction

Cells dependent on ECM anchorage require substrate adhesion before normal function is initialized. If adhesion is disrupted or prevented, the cell will die through a specialized apoptosis, anoikis. It is the ability of cells, such as osteoblasts to adhere that allows signalling from the ECM to the nucleus via integrins, cytoskeleton, and molecular cascades (G-proteins, kinases, ions). These events ultimately control proliferation and new tissue formation.

The conversion of physical signals, such as contractile forces or external mechanical perturbations, into chemical signaling events is a fundamental cellular process that occurs at cell–extracellular matrix contacts, known as focal adhesions. Focal adhesions (FAs) are peripherally located adhesion sites associated intimately with the actin cytoskeleton that form adhesion plaques of clustered transmembrane receptors at points of cellular adhesion. FAs contain structural and secondary signalling molecules crucial to cell adhesion and function. Conversely, materials that promote osteoblast specific adhesion may enhance functional differentiation. Materials that promote osteoblastic differentiation upregulate osteospecific genes resulting in the neogenesis of mineralized matrix,bony tissue formation, and deposition. The manipulation of a substrate's topography to affect its adhesive potential and the ability of cells to adhere at the cell–substrate interface may be viewed as an approach to direct and organize the adhesion and differentiation of migrating cells in bone repair.[Biggs et al., ,2007, Cavalcanti-Adam, et al., 2007]

The forces applied to adhesion sites are not determined exclusively by the cellular contractile machinery. An additional important factor is the mechanical nature of the underlying substrate. When cells are attached to a soft flexible substrate, which can be easily deformed, the tension acting on the adhesion plaques may be smaller than the force needed to sustain the adhesion site and the attached stress fibers. Consequently, the typical dimensions of focal adhesions formed with such substrates are considerably smaller than those formed following attachment to a rigid surface This ability to discriminate between soft and rigid substrate enables cells to become oriented when they sense a gradient in substrate rigidity and move along the substrate in the direction of higher rigidity (a phenomenon known as durotaxis [Bershadsky et al., 2003].

5.2. Mitochondrial Bioenergetics: MMP

Robust demands for energy are placed on osteoblasts during the production of a mineralized matrix, which provides both structural support and a calcium reservoir. Mitochondria are by far the main producers of ATP in eukaryotic aerobic cells. In addition mitochondria play other important roles in cell physiology and pathology, including participation in ions homeostasis, regulation of the cell redox state (including reactive radical biology), transport of metabolites, including import of proteins synthesized in the cytosol, lipid and amino acid metabolism, cell death. These important functions are highly dependent

on the electrochemical transmembrane potential (proton motive force: $\Delta p = \Delta\Psi - 2.3(RT/F)\Delta pH$), a physico-chemical parameter consisting of two components: $\Delta\Psi$, the total transmembrane electrical potential (voltage gradient), and ΔpH, the proton gradient that is physiologically generated across the inner mitochondrial membrane by the respiratory chain activity. The mitochondrial membrane potentials (MMP) reflect the functional status of mitochondria within cells**,** is key to the maintenance of energy within cells and drives the synthesis of ATP. The accurate assessment of the MMP within cells is central to understanding the role of mitochondria in cells. [Zhang et al., 2001; Solaini et al., 2007; Salido et al., 2007].

For the combined backscattered and fluorescence study of the living osteoblasts, an in situ technique in which mitochondria within the intact cells could be probed, cells were stained with the ratiometric cationic dye JC-1 (that exhibits potential-dependent accumulation in mitochondria). (Figures 10, 11). At low membrane potentials, JC-1 continues to exist as a monomer and produces a green fluorescence (emission at 527 nm). At high membrane potentials, JC-1 forms “J-aggregates" (emission at 590 nm) and produces a red fluorescence.

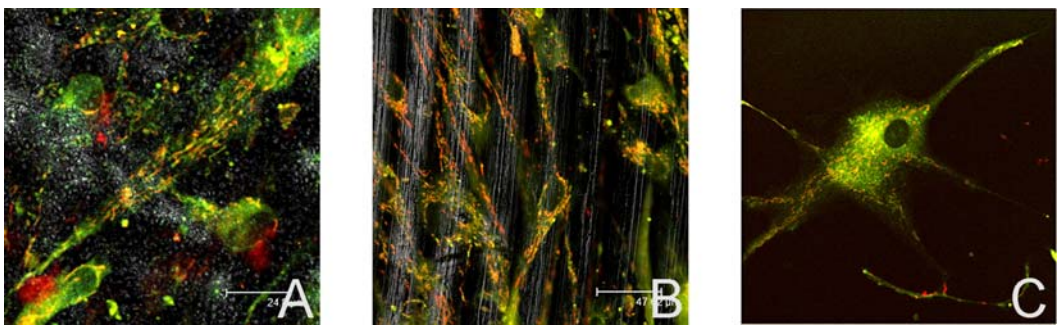

Figure 10. Human NHOst stained with the ratiometric dye JC1. In red, J-aggregates, indicative of high MMP. Green staining represents low MMP zones. Cell shape and highly energized mitochondrial distribution changes depending on the underlying surface. A. Cells growing on the rough surface. Magnification 63x, zoom 1.2. B. Cells growing on the machined surface. Magnification 63x, zoom 1. C. Control cells grown on glass. Magnification 63x, zoom 1.

5.3. Image Collection and Analysis for $\Delta\psi_m$

The cells and disks were simultaneously visualized using a Leica TCS-SL confocal microscope equipped with a 63.0 x 1.30 glycerol objective, under the conditions described above, allowing simultaneous acquisition of surface reflectance and JC1 stained mitochondria. JC-1 was excited at 490 nm and the emission fluorescence was collected in TRITC (590 nm) and FITC (530 nm) channels simultaneously.

For quantitative analysis, at least 120 regions of interest (ROIs) were selected in each group to quantify changes in $\Delta\psi_m$. All of the ROIs are cells selected under the following criteria: well defined limits, clear identification of nucleus and absence of intersection with neighbouring cells. Size, number of pixels and fluorescence intensity in the red (high membrane potential) and green (low membrane potential) channels in each ROI were calculated. All samples were exposed to laser for a time interval not higher than 5 min to avoid photobleaching.

The excitation beam splitter selected was a DD 488/543. The laser was set to the lowest power able to produce a fluorescent signal. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible. Offset was maintained at 0. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 1024 x1024. Series were acquired in the xyz mode. Both reflected light and fluorescence were collected along the same optical axis and separated immediately prior to entry into respective photomultipliers (PMT) thus allowing discrimination of fluorescence from the scattered light signal.

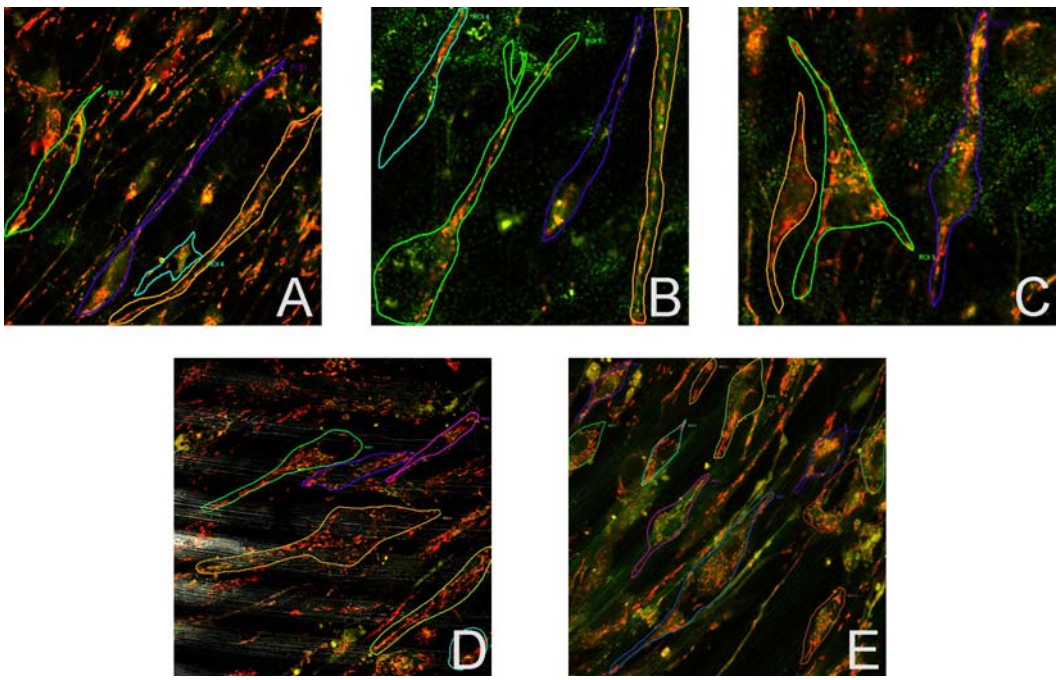

Figure 11. JC1 stained human osteoblasts (NHOst). As shown in A,B,C, highly energized mitochondria clustered and polarized to focal contact zone. D,E: Osteoblasts grown on machined surface with red mitochondria distributed along the cell. As presented in all images, ROIs have been selected for MMP quantification and statistical assays. Magnification 63x, zoom 1.

As shown in the images, the focal complexes and FA that are formed during spreading are accompanied by mitochondrial clustering, which could help to cytoskeletal organization acting as surface-sensing entities that control, locally and globally, adhesion-mediated signaling and coordinate the adhesive and migratory process. The mitochondrial features observed reveal how cells can sense and integrate multiple chemical and physical features of the underlying adhesive substrate.

CLSM combining fluorescence and backscattered modes, thus, has revealed to be a useful tool for investigating the dynamics of biological interactions among these components and the mechanisms by which specific structural and mechanical properties of the three dimensional extracellular matrix microenvironment influence cell behavior. CLSM image combining the fluorescence and reflection modes helped us to illustrate cell-surface interaction and to demonstrate that osteoblasts assume distinct morphologies depending on the architectural features of their substrate.

This approach may contribute to a better understanding of the processes involved in the integration of oral implants. Despite the immense success of oral implants and implants in general, implant performance still needs to be improved, especially for patients with an unfavorable prognosis because of impaired bone or soft tissue repair due to underlying diseases.

6. CONCLUSION

Osseointegration of implants is crucial for the long-term success of oral implants. Recent research has suggested that bones display an extraordinary adaptative behaviour towards changing mechanical environment, which is often regarded as phenotype plasticity. Furthermore, the peri-implant tissue formation and mineralization by osteoblasts are strongly dependent on the local mechanical environment in the interface zone.

Mitochondria are diverse in form and function, and they participate in various critical cell functions in addition to bioenergetics. There is a direct correlation between the energized state of mitochondria and the $\Delta\psi_m$ when analyzed in isolated mitochondria

APPENDIX

Methodological Approaches

A.1. Test Substrates

Prefabricated 2 cm x 1.5 mm disks with identical surface topography than the desired dental implants were prefabricated for the experiments. Before use, the disks were immersed in 100% ethanol for 10 min, air dried, and exposed under u.v. light for 30 min on each side, and finally rinsed in endotoxin free phosphate buffered solution and deposited on small sterile Petri dishes prior to cell seeding. Tissue culture Willco® plates with 0.17 mm glass bottom were used as the control surface.

For the adequate examination of disks in the confocal microscope, one holed polycarbonate slides were designed in our laboratory and fabricated by means of control precision systems. (Mecaprec, Cadiz, Spain) The device designed allows us to carefully preserve cellular integrity and also avoids the problem of device thickness interference with focus distance, as the disk is inserted into the slide prior to mounting with appropriate mounting media and a 0.17 mm coverslip.

A.2. Cell Culture

Norman human osteoblastic NHOst® cells (Cambrex, Walkersville, MD, USA) were seeded at a density of 5000 cells/ cm2 and incubated in Osteoblast Growing Medium, OGM, (Cambrex, Walkersville, MD,USA) containing 10% fetal bovine serum (Cambrex, Walkersville, MD, USA), 1% gentamycin sulphate/amphotericin B (Cambrex, Walkersville, MD, USA) and 1% ascorbic acid (Cambrex, Walkersville, MD, USA), as recommended by suppliers, at 37°C and 5% CO_2 until the experiments were started. Growth medium was changed every day after seeding. Before the cells became 80% confluent they were subcultured with 2 ml of 0.25 mg/ml trypsin EDTA warmed to 37°C (Cambrex, Walkersville,

MD, USA) after rinsing with 5 ml Hepes-BSS (Cambrex, Walkersville, MD, USA) at room temperature. Once cells were detached, trypsin EDTA was neutralized by adding 4 ml of trypsin neutralizing solution (Cambrex, Walkersville, MD, USA). Harvested cells were seeded on the different surfaces at a density of 5000 cells/cm2 and immunostained after 48 h. Growth medium was changed every day until the experiment was finished. NHOst cells are assured for experimental use for ten population doublings, which were not exceeded during the assay. Tissue culture Willco® (WillCo Wells, Amsterdam, the Netherlands) wells with 0.17 mm glass bottom were used as the control surface.

A.3.Cytoskeletal Organization

At the end of the specific culture time, cells were washed twice with prewarmed phosphate –buffered saline, (PBS), pH 7.4, and fixed with 3.7% paraformaldehyde (PFA) solution in PBS for 10 min. at room temperature and washed twice with prewarmed PBS. The cells were then permeabilized with 0.1% Triton x-100 (Sigma's Louis, Missouri, USA) for 5 min and washed twice with prewarmed PBS. To reduce non-specific background staining 1% bovine serum albumin (BSA) in PBS was added to the surfaces for 20 min and cells were immunostained for 20 min. with rhodamine phalloidin, 12.5µl of methanolic stock solution (Sigma's Louis, Missouri, USA) in 500 µl PBS for each sample. After discarding staining solution, disks were rinsed with prewarmed PBS three times prior to mounting with Vectashield® (Vector Labs. Burlingame CA, USA) and 0.17 mm coverslip in one holed polycarbonate slide devices specially designed in our laboratory and fabricated by means of control precision systems (Mecaprec, Cadiz, Spain)

A.4. Mitochondrial Permeability Potential

Cells were cultured in Willco® wells with glass bottom as control group for the assay, and on the different disks surfaces provided, not exceeding a final number of 10^6 cells/ml, and after discarding culture medium, 1x MitoPT® Mito PT™®, Immunohistochemistry Technologies, Bloomington, MN, USA), staining solution obtained from a 100x stock was added to the wells, 0.5 ml per well. Cells were then incubated at 37º for 15 min in a CO_2 incubator and, after discarding medium, washed twice with 1–2 ml of assay buffer warmed to 37º. After wash is discarded, a drop of assay buffer was added to the specimens prior to immediate examination in the inverted confocal microscope Leica TCS SL (Leica, Darmstaad, Germany), equipped with an HCX PL APO CS 63.0 x 1.30 glycerol immersion objective, with an incubation system consisting in a cube that completely covers the microscope and allows us to keep cells at 37º in a controlled atmosphere with a mixed air/CO_2 flow of 4 l/h and 5% CO_2 during image collection and analysis.

A.5. Statistical Analysis

The statistical analysis was performed with SPSS program. A one-way ANOVA analysis was used to compare the mean values for cell areas and red pixels intensity, standard deviation and skewness. The normality of the groups was contrasted with Kolmogorov-Smirnov test and the variances homogeneity with Cochran's C test. Post-hoc contrasts, HSD test of Tukey, were carried out to detect the differences between groups. A value of $p \leq 0.05$ was considered significant.

REFERENCES

Albrektsson, T., Wennerberg, A. (2005), The impact of oral implants: past and future, 1966-2042. *J.Can.Dent.Assoc.*, 71, 327.

Bershadsky, A.D., Balaban, N.Q., Geiger, B. (2003). Adhesion-dependent cell mechanosensitivity. *Annu. Rev. Cell Dev. Biol.,* 19, 677–695.

Biggs, M.J.P., Richards, R.G, Gadegaard, N., Wilkinson, C.D.W, Dalby M.J. (2007). Regulation of implant surface cell adhesion: characterization and quantification of s-phase primary osteoblast adhesions on biomimetic nanoscale substrates. *J. Orthop. Res.,* 25, 273–282.

Branemark, P.I., Zarb, G.A., Albrektsson, T. (1999). Tissue Integrated Prostheses Osseointegration in Clinical Dentistry. Barcelona, Spain: Editorial Quintessence.

Cavalcanti-Adam, E.A., Volberg, T., Micoulet, A., Kessler, H., Geiger, B., Spatz, J.P. (2007). Cell Spreading and Focal Adhesion Dynamics Are Regulated by Spacing of Integrin Ligands. *Biophys. J.,* 92, 2964–2974.

Cícero Dinato, J., Daut Polido, W. Implantes Oseointegrados: Cirugía y Prótesis. (2003). Sao Paulo, Brasil. Ed. Artes Médicas Latinoamérica.

Cohen, M., Joester, D., Geiger, B., Addadi, L. (2004). Spatial and Temporal Sequence of Events in Cell Adhesion: From Molecular Recognition to Focal Adhesion Assembly. *Chem. Bio. Chem.,* 5, 1393–1399.

Dalby, M.J. (2005).Topographically induced direct cell mechanotransduction. *Med. Eng. Phys,* 27,730-742.

Davies, J.E (2003). UNderstandig peri-implant endosseous healing. J. Dent. Educ. 67(8):932-949

Davies, J.E. (1996). In vitro modeling of the bone/implant interface. *Anat.Rec.*, 245, 426-445.

Degasne, I., Basle, M.F., Demais, V., Hure, G., Lesourd, M., Grolleau, B., Mercier, L., Chappard, D. (1999). Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. *Calcif.Tissue Int.*, 64, 499-507.

Diener, A., Nebe, B., Lüthen, F., Becker, P., Beck, U., Neumann, H.G., Rychly, J. (2005). Control of focal adhesion dynamics by material surface characteristics. *Biomaterials,* 26, 383-392.

Gallucci, G.O., Mavropoulos, A., Bernard, J-P., Belser, U.C. (2007). Influence of inmediate implant loading on peri-implant soft tissue morphology in the endentulous maxilla. *Int J. Oral. Maxillofaci. Implants*.22; 595-602.

Harris, L.G., Patterson, L.M., Bacon, C., Gwynn, I.,Richards, R.G. (2005). Assessment of the cytocompatibility of different coated titanium surfaces to fibroblasts and osteoblasts. *J.Biomed.Mater.Res.A.*, 73, 12-20.

Hibbs, AR. Confocal Microscopy for Biologists. New York: Kluwer; 2004.

Joos, U., Meyer, U. (2006).New paradigm in implant osseointegration, *Head Face.Med.*, 2, 19.

Karp, G. (2007). Biología Celular y Molecular. Conceptos y experimentos. (4th), Méjico, D.F.,Mejico: Mc Graw-Hill Interamericana.

Keller H.E. (2005). Objective lenses for confocal microscopy. In: Pawley J.B. editor. Handbook of biological confocal microscopy... New York; Springer; pp: 145-160

Keller, J.C., Schneider, G.B., Stanford, C.M., Kellogg, B. (2003). Effects of implant microtopography on osteoblast cell attachment. *Implant.Dent.*, 12, 175-181.

Kikuchi, L., Park, J. Y., Victor, C., Davies, J.E. (2005). Platelet interactions with calcium-phosphate-coated surfaces. *Biomaterials*, 26, 5285-5295.

Lim, J.Y., Dreiss, A.D., Zhou, Z., Hansen, J.C., Siedlecki, C.A., Hengstebeck, R.W., et al (2007). The regulation of integrin-mediated osteoblast focal adhesion and focal adhesion kinase expression by nanoscale topography. *Biomaterials*; 28,1787-1797.

Linez-Bataillon, P., Monchau, F., Bigerelle, M., Hildebrand, H.F. (2002). In vitro MC3T3 osteoblast adhesion with respect to surface roughness of Ti6Al4V substrates. *Biomol. Eng*, 19, 133-141.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, S. L., Darnell, J. (2005). Biología Celular y Molecular (5th). Buenos Aires, Argentina. Editorial Médica Panamericana.

Meyer, U., Büchter, A., Wiesman, H.P., Jung, Y.C., Jones, D.B. (2005). Basic reactions of osteoblasts on structured material surfaces. *Eur. Cell Mat.,* 9, 39-49.

Okumura, A., Goto, M., Goto, T., Yoshinari, M., Masuko, S., Katsuki, T., Tanaka, T. (2001). Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2). *Biomaterials* 22, 2263-2271.

Papalexiou, V., Novaes, A.B.Jr., Grisi, M.F., Souza, S.S., Taba, M.,Jr., Kajiwara, J.K. (2004). Influence of implant microstructure on the dynamics of bone healing around immediate implants placed into periodontally infected sites. A confocal laser scanning microscopic study. *Clin.Oral Implants. Res.*, 15, 44-53.

Park, B.S., Heo, S.J., Kim, C.S., Oh, J.E., Kim, J.M., Lee, G., et al. (2005). Effects of adhesion molecules on the behavior of osteoblast-like cells and normal human fibroblasts on different titanium surfaces. *J.Biomed.Mater.Res.A.,* 74, 640-651.

Popat, K.C., Leoni, L., Grimes, C.A., Desai, T.A. (2007). Influence of engineered titania nanotubular surfaces on bone cells. *Biomaterials* ,28, 3188-3197.

Riveline D, Zamir, E., Balaban, N.Q., Schwarz, U.S., Ishizaki, T., Narumiya, S. et al. (2001). Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mdia1-dependent and rock-independent mechanism. *J Cell Biol*, 153, 1175–1185.

Rouahi, M., Champion, E., Hardouin, P., Anselme, K. (2006). Quantitative kinetic analysis of gene expression during human osteoblastic adhesion on orthopedic materials. *Biomaterials*, 27, 2829–2844.

Sader, M.S., Balduino, A., de Almeida, S.G., Borojevic, R. (2005). Effect of three distinct treatments of titanium surface on osteoblast attachment, proliferation, and differentiation. *Clin.Oral Implants.Res.*, 16, 6, 667-675.

Salido, M., González, J.L., Vilches, J. (2007). Loss of mitochondrial membrane potential is inhibited by bombesin in etoposide-induced apoptosis in PC3 prostate carcinoma cells. *Mol. Cancer Ther.* 6, 1292-1299.

Schwarz, U.S., Bischofs, I.B. (2005). Physical determinants of cell organization in soft media. Med. Eng. Phys., 27, 763–772.

Shah A.K., Sinha R.K., Hickok N.J., Tuan R.S. (1999). High resolution morphometric analysis of human osteoblastic cell adhesion on clinically relevant orthopedic alloys. *Bone* 245, 499-506.

Solaini, G., Sgarbi, G., Lenaz, G., Baracca, A. (2007). Evaluating Mitochondrial Membrane Potential in Cells. *Biosci. Rep.,* 27, 11–21.

Tan, W., Sendemir-Urkmez, A., Fahrner, L.J., Jamison, R., Leckband, D., Boppart, S.A. (2004a). Structural and functional optical imaging of three-dimensional engineered tissue development. *Tissue Eng*; 10:1747-1756.

Tan, W., Vinegoni, C., Norman, J.J., Desai, T.A., Boppart, S.A. (2007). Imaging cellular responses to mechanical stimuli within three-dimensional tissue constructs. *Microsc. Res. Tech.,* 70, 361-371.

Vilches, J., Vilches-Perez, J.I. , Salido, M. (2007). Cell-surface interaction in biomedical implants assessed by simultaneous fluorescence and reflection confocal microscopy. In: Mendez Vilas, editor. *Modern Research and Educational Topics in Microscopy*. Vol I pp. 60-67 Badajoz, Spain: Formatex.

Voytik-Harbin, S.L., Roeder ,B.A., Sturgis, J.E., Kokini, K., Robinson, J.P. (2003). Simultaneous mechanical loading and confocal reflection microscopy for three dimensional microbiomechanical analysis of biomaterials and tissue constructs. *Microsc. Microanal,* 9,74-85.

Zhang, H., Huang, H.M., Carson, R.C., Mahmood ,J., Thomas, H.M., Gibson, G.E. (2001). Assessment of Membrane Potentials of Mitochondrial Populations in Living Cells. *Analyt. Biochem.,* 298, 170–180.

Zimerman, B., Arnold, M., Ulmer, J., Blümmel, J., Besser, A., Spatz, J.P., Geiger B. (2004a). Formation of focal adhesion-stress fiber complexes coordinated by adhesive and non-adhesive surface domains. *IEE Proc. Nanobiotechnol.,*151, 62-66.

Zimerman, B., Volberg, T., Geiger, B. (2004b). Early Molecular Events in the Assembly of the Focal Adhesion-Stress Fiber Complex during Fibroblast Spreading. *Cell Mot.Cytoskeleton,* 58,143–159.

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Chapter 4

DENTAL MATERIALS IN DAILY PEDODONTICS CLINICAL PRACTICE

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ABSTRACT

The advent of adhesive materials has allowed smaller preparations to be made, thus preserving sound tissues, in line with the Minimum Intervention (MI) philosophy. One needs to bear in mind that retentive preparations, with straight angles and convenient shape were necessary when the best restorative material available was amalgam, with no adhesive ability. Nevertheless, the option to restore and the type of material to use continue to be a matter of concern. Restorative dentistry undoubtedly plays a role in recovering function, aesthetics and allowing biofilm control by the patient/family, but healing the disease cannot be credited solely to the restoration, as it is associated with the control of etiological factors. Moreover, restorations in the oral environment are exposed to stresses of different origins that limit their longevity by interfacial degradation. Restoration failures lead to replacement that implies further removal of dental structure, causing repetition of the restorative cycle. Thus, the decision to restore and the choice material involve the determining the patient's caries activity, immediate requirements and the notion of what the patient is able to receive in terms of dental procedures. In this sense, and considering the short biological course of primary dentition, materials once considered temporary, such as glass ionomer cement (GIC), can be used as definitive or even as an intermediate stage in the treatment plan. GIC presents chemical adhesion to the tooth structure, which is believed to be beneficial in terms of resistance to degradation, expansion coefficient similar to tooth structure, fluoride release and uptake, and GIC is especially indicated for temporary dentition. There is also the trend of incorporating GIC components into adhesive systems, with the intention of adding chemical adhesion to the conventional bonding mechanism—the glass ionomer adhesive system. Clinical time in the dental chair is also a problem where children are concerned, and it is important to develop techniques that speed up and facilitate the procedures. Therefore, the simplified adhesive systems play a role in these cases, and must be studied. Along the same lines, to reduce chair time, resin sealants can act therapeutically, when primary teeth have small cavities that reach dentin. In these cases, carious dental

tissue is not removed; all tissue is preserved. Since it is known that "the seal is the deal", sealing the cavity will reduce bacterial counts and will probably stop the lesion.

1. INTRODUCTION

Caries lesion is the consequence and clinical sign of caries disease. Therefore, restoring the clinical sign does not mean cure of the disease, but only the return to normality in esthetic and functional terms. Cure or control of the disease is achieved by changing attitudes as regards diet and oral hygiene.

The **main role of restoration**, in this context, is to enable the site to be adequately cleaned, thereby preventing biofilm accumulation, which is responsible for caries lesion progression. The restoration may also help to reduce the sensitivity cause, which is very important, since it could affect the quality of life of patients with dental lesions.

In order to **choose the best restorative material**, many aspects must be taken into account, among them, the time the tooth will remain in the oral cavity, especially in the case of primary teeth; the patient's caries activity; this patient's cooperation during attendance; the type of substrate and cavity to be restored, among others.

Generally speaking, the adhesive systems associated with resin composite and glass ionomer cements have been the restorative materials of choice for use in pediatric dentistry.

2. ADHESIVE SYSTEMS ASSOCIATED WITH RESIN COMPOSITES

The majority of protocols for bonding to dentin involve acid etching of the surface, which removes or modifies the smear layer and decalcifies the subjacent dentinal structures. The peritubular dentin is partially demineralized, widening the dentinal tubule entrances, which take on a funnel-like appearance. Concomitantly, the intertubular dentin is etched, exposing the collagen matrix. The application of resinous monomers results in the formation of a hybrid layer (Nakabayashi et al., 1982) or interdiffusion zone (Van Meerbeek et al., 1992). The hybrid layer or interdiffusion zone consists of resins monomers interlaced with the collagen fibers exposed by etching which, once polymerized, compose this hybrid structure that provides micromechanical bonding.

Hybrid layer formation depends on appropriate conditions (Van Meerbeek et al., 1992) and compliance with the protocol at every step:

- dentin etching (demineralization with the use of acid solutions), followed by exposure of the reticulated collagen fibers);
- maintaining the structural integrity of the collagen fibers; that is, preventing them from being denatured;
- application of bifunctional monomers (hydrophilic-hydrophobic) that are capable of interpenetrating the reticulated collagen fibers of the etched subsuperficial dentin, and being polymerized there.

The application of phosphoric acid on dentin dissolves the hydroxyapatite crystals of the surface and subsurface, leaving reticulated collagen fibers in a thickness of approximately 2.0 micrometers (0.2 mm) for subsequent infiltration of the adhesive agent (Gwinnett, 1994; Pashley et al., 1993; Van Meerbeek et al., 1992).

The aim of using acid should be to demineralize around 2 micrometers, sufficient to enable the hydrophilic monomers to permeate this depth completely and perfectly, and also to leave the hydroxyapatite crystals of the basal dentin unaltered, thus avoiding the discrepancy between the zone of demineralized dentin and that of penetration of the resinous monomers, and the possibility of compromising the unprotected collagen fibers (Nakabayashi et al., 1992). With 37% phosphoric acid as the etching agent, an application time of between 5 and 15 seconds is sufficient for achieving demineralization of around 1.0 to 2.0 micrometers. The ideal hybrid layer should be thick enough to allow the mechanical misalignment of the adhesive around the exposed collagen fibrils, but the demineralization zone must not be excessively deep. Furthermore, the collagen fibrils exposed in the portion of demineralized dentin allow nanoinfiltration, creating zones susceptible to hydrolytic and enzymatic degradation with the passage of time, compromising the longevity of the restoration (Sano et al., 1995; Pashley et al., 2004; Hashimoto et al., 2000b). The discrepancy between the demineralization zone and that of resinous monomer penetration may result in low bond strength values for primary tooth dentin (Burrow et al., 2002; Uekusa et al., 2006) due to their greater reactivity to acid etching (Nör et al., 1996; 1997). In this sense, a reduction in etching time (Sardella et al., 2005) and the use of weaker acids have been proposed for primary dentinal substrate (Nör et al., 1996). Thus, in primary dentin, an etching time of between 7-10 seconds with 37% phosphoric acid is encouraged, and the acid should therefore first be applied on the enamel and then on the dentin.

The quantity of water present in the reticulated collagen fibers has an influence on the bond quality. Excessive post-washing drying after acid etching would cause “collapse” or contraction of the reticulated collagen fibers (Pashley et al., 1993; Gwinnett, 1994) as a result of the loss of water that occupied the interstitial spaces in the collagen network, which were left by the hydroxyapatite crystals that were dissolved by the action of the acid. This phenomenon diminishes permeability by the monomers of the adhesive system, compromising bond strength. Therefore, for bonding to dentin, a humid surface becomes almost a necessity. The degree if humidity of the etched dentin surface has a strong influence on the application technique; add this to the difficulty of standardizing the degree of humidity required for humid bonding, and this makes the clinical procedure very sensitive to small variations. This difficulty arises because there is no objective way to establish this degree of humidity, so the wide variability of subjectivity takes over, and reflects a serious and important clinical difficulty (Perdigão et al., 1999; Miyazaki et al., 2000; Perdigão et al., 2001b; Shono et al., 1999).

Different techniques for drying after washing have been proposed, with a view to maintaining a humid surface: air jets, air jet for 3 seconds at 20 cm (Tay et al., 1996), absorbent paper, cotton wool, and microbrushes (Goes et al., 1997). With these maneuvers, one endeavors to achieve a visibly humid surface without an excess of water, characterized only by the shiny aspect. The use of absorbent paper and cotton wool has shown to be more effective for this purpose (Goes et al., 1997).

In the case of over-drying, the condition can be reverted by the application of water, maintaining it on the dried dentin surface for at least 30 seconds before applying the

adhesives (Perdigão et al., 2001b; Tay et al., 1997; Gwinnett et al., 1994). The manner of action of water in this process of re-hydration and re-expansion consists of it high diffusion capacity, due to the diminutive size of its molecule that is capable of rupturing the bridge-type hydrogen bonds that maintain the fibers in a state of collapse (Perdigão et al., 2001b; Carvalho et al., 1996; Pashley et al., 2002; Pashley et al., 2000).

Basically, adhesive systems have three components in their composition: a) etching agent, b) primer and c) adhesive or bond that can be presented separately or associated. The association of components was idealized with the idea of reducing the number of operative steps and improving the usability of the adhesive systems, and diminishing the technical sensitivity of each clinical step. Initially, the primer and adhesive were fused into a single component, and were denominated Single Bottle systems, with the phosphoric acid etchant still provided separately (Burrow et al., 1999; Van Meerbeek et al., 2001). These systems, denominated the 5th generation, present results similar to those of their previous versions, however, they demand successive applications and light activations, and do not effectively reduce clinical time (Perdigão et al., 1995), so that the classification as *one-step adhesive systems* proposed by some authors is being questioned.

Another form of associating components was joining the primer to the etching agent, thus giving rise to the self-etching systems that unite acids, which modify the smear layer and incorporate it into the hybrid layer, with the primer, or with the adhesive itself, with the goal of simultaneously demineralizing the collagen fibers, and the adhesive interpenetrating among them; thus diminishing the technique sensitivity and improving usability. These systems have made the clinical procedures less critical, especially with regard to the difficult question of maintaining the degree of dentinal humidity, as occurs with the present day adhesives that require previous etching with phosphoric acid.

The systems denominated self-etching can be classified as follows: 1) self-etching primers and 2) all-in-one adhesives.

By self-etching primers, one understands those systems in which the primer does indeed etch the enamel and dentin and its role is to prepare the dental surface for bonding, followed by the application of the bond. These systems are characterized by the separate application of the primer and the adhesive (Tay, Pashley, 2001). These systems were designated as the 6th generation adhesive, and are presented in two bottles applied separately.

The second group, that of the self-etching adhesives for single application, (all-in-one), are characterized by preparation of the mixture between the primer and adhesive, immediately before application, followed by their polymerization. They are also known as the 7th generation adhesives, and the form of presentation is normally in two bottles that have to be mixed before application. There are some systems that use blisters that maintain the components separated to be mixed at the time of application.

There are basically two types of self-etching adhesives: moderate and strong (Van Meerbeek et al., 2001). The strong self-etching adhesives have a very low pH (<1) and have a bond mechanism and morphological structure at the interface with dentin that is similar to that produced by the total acid etching adhesives. The moderate self-etching adhesives (pH around 2) partially dissolve the dentin surface so that a substantial number of hydroxyapatite crystals remain in the hybrid layer. Functional monomers can thus chemically interact with this hydroxyapatite (Yoshida et al., 2000). This double bond mechanism (micromechanical and chemical) can be advantageous in terms of durability (De Munck et al., 2005).

The action mechanism of self-etching adhesives is thus influenced by the pH, and concentration and types of acidified monomers. Tay and Pashley (2001) explained that the concentration of 20% of phosphated monoester in the product Clearfil SE Bond and 80% of phosphated diester in Prompt L-Pop (present day Adper Prompt – 3M ESPE), modified the pH of these products, causing the acidity to be duplicated (pH 2.0 and 1.0, respectively). This variation causes the product Clearfil SE Bond to have a moderate action on the smear layer and smear plug and form a thin hybrid layer, to the order of 0.5 micrometers. Whereas, Prompt L-Pop results in complete dissolution of the smear layer and demineralization to the order of 2.5 to 5.0 micrometers. It is interesting to note that even without washing, the power of dissolution of products with lower pH, are similar to that of phosphoric acid, which has a pH of around 0.6. A demineralization thickness similar to that obtained with phosphoric acid etching was reported when using the Adper Prompt system in primary teeth, the results of the bond strength with this system being inferior to those obtained with other self-etching system with higher pH, which produced thinner demineralization zones (Bolaños-Carmona et al., 2008).

Details such as the need for mixture, application sequence, number of layers, action time, forms of excess removal, light polymerization time, are different for each product. Thus it is mandatory to read the descriptive leaflet before proceeding with application of the material, as each product has technical peculiarities that have a great deal of influence on the results.

One of the most interesting details with regard to the application techniques refers to the need for less concern about the degree of hydration of the dentinal surface, since the washing and subsequent drying steps are not performed, and thus one avoids the possible problems previously discussed.

As far as the effectiveness of the self-etching adhesives is concerned, laboratory studies have pointed out results with values of bond strength to dentin close to those achieved with the adhesives that used to total etching technique with phosphoric acid (Frankenberger et al., 2001; Inoue et al., 2001; Miyazaki er al., 2001; Toledano et al., 2001).

As regards clinical effectiveness, Casagrande et al. (2006) followed-up restorations in primary molars after clinical function for a period of 15 to 17 months with the Clearfil SE Bond self-etching system, and verified bond strength values similar to those found *in vitro* for non-aged restorations. Furthermore, there was no sign or symptom of failure, such as sensitivity or pulp lesion. The success achieved with these self-etching adhesive systems is credited to the complete impregnation of resinous monomers throughout the depth of the etched dentin, which would avoid the problem of unprotected collagen fibers being subject to degradation (Hashimoto et al., 2000a).

3. GLASS IONOMER CEMENTS

Glass ionomer cements (GIC) were developed thanks to the search for a material that would present satisfactory esthetic properties and physical-chemical bonding to dental tissues. When examining cements prepared with a mixture of silicate glass and an aqueous solution of polyacrylic acid, Wilson (1968) began the first studies with the product that would eventually be denominated glass ionomer cement (Wilson, Kent, 1972). The composition of glass ionomer cements is complex and varied, but all have the principle of an acid-based chemical

reaction. The basic component is represented by calcium aluminum-silicate glass containing fluorides and the acid component by polyacid. The radiopacity of the material is obtained at the expense of incorporating elements such as strontium, barium and zirconium. At present, glass ionomer cements have been presented with a series of modifications to their basic composition with the aim of improving the properties of the material (Leinfelder, Kurdziolek, 2001; Navarro, Pascotto, 1998).

The setting mechanism by which conventional glass ionomer cements are processed involves a complex chemical reaction in which polyacid (generally polyacrylic acid) attacks the aluminum silicate glass particles. With the release of hydrogen ions from the polyacrylic acid, the calcium, aluminum and fluoride ions are released from the glass starting an initial reaction of jellification and hardening of the cement with the preferential formation of calcium polyacrylate (Navarro, Pascotto, 1998). This phase is known as initial setting, having the clinical aspect of loss of surface shine, which demonstrates this phase. After a few hours, the formation of a silicone network in the material occurs, improving resistance after initial hardening, lasting for approximately 24 hours. This phase is known as final setting. Associated with this phase, the polyacrylate chain displaces phosphate ions from the dental surface.

Glass ionomer cements can be classified in two ways: with regard to their nature or to their use. In the first classification we have the **conventional cements** (composed of particles of glass powder and polyalkenoic liquids), those **reinforced with metals** (mixture of conventional powder with particles of amalgam alloy or silver sinterized with glass particles) and those that are **resin modified** (part of the polyalkenoic acid is replaced by hydroxyethyl methacrylate)(Navarro, Pascotto, 1998).

Whereas, the classification of MOUNT (1994) would be: **Type I** (cements for cementation of incrustations, crowns, dentures and orthodontic devices), **Type II** (cements for restorations), **Type III** (cements for lining or base and for sealing fossas e cicatrizes), and **Type IV** (resin modified ionomeric cements, contemplating all the indications).

Recently, chemical setting glass ionomer cements were introduced, indicated for restorations in locations with masticatory loads, which present superior physical properties, due to the increase in load and the power-liquid ratio. These materials (e.g., Fuji IX GP – GC Corp; Ketac Molar Easy Mix – 3M ESPE) present a fast setting reaction (by the addition of tartaric acid), which has contributed significantly to the reduction in sensitivity to contamination. They are indicated for restorations in primary and permanent teeth, particularly for performing Atraumatic Restorative Treatment (ART). These materials are also known as "high viscosity materials" (Berg, 2002).

The chemical composition of resin modified glass ionomer cements, also known as hybrids, varies according to the material. In general, they incorporate light activated resinous components, such as the methacrylate, HEMA and liquid photosensitizing groups. These materials have a double setting reaction and in some situations, triple. The mixture of their components begins a classical acid-based reaction, as occurs in conventional glass ionomer cements, associated with light polymerization of the methacrylate groups. In some materials yet a third reaction of chemical polymerization of the free radicals of the methacrylate groups occurs. When the reaction starts, a network is formed, in which the ionomer matrix (hydrogel) unites with the resin. The combination of these phenomena result in lower sensitivity to the action water during the setting reaction, associated with control of the setting and working time, the main advantage of this type of material. In order to achieve the best

physical properties, it is important to obtain a high powder/liquid ratio. These materials have been used very successfully in pediatric dentistry over the last few decades.

A new generation of GIC has recently been launched on the market, which allies the knowledge of nanotechnology, by adding nanoparticles of resin to the ionomeric material. This new material is presented in a paste-paste form, which greatly facilitates dosage and manipulation, presents dual and chemical setting, and is light polymerizable. At the moment there is little literature available about this material, and mechanical and bond strength tests are being conducted.

The development of GIC has greatly improved these characteristics. The materials in which polyacrylic acid is presented for addition to the liquid are more difficult to manipulate. This is more visible in the high viscosity materials, as it is necessary to incorporate a larger quantity of powder in the same quantity of liquid. It is necessary to follow the manufacturers' recommendations at all times, as regards dosage, and alterations in proportion on one's own account are completely contraindicated. The powder dosing spoon must be level, in order to standardize the quantity. For this purpose, the spoon should be scraped against the side of the flask, or use a spatula to remove the excess. The flask of liquid must be in the vertical position in order to dose a full drop. To increase the wettability property, the ideal is to reduce the angle of contact between the liquid and manipulation block with a simple maneuver of "thinning" the drop. With this simple maneuver, incorporation of powder into the liquid is facilitated. Soon afterwards, the ideal is to separate the powder into two parts, taking the first into contact with the liquid for 15 approximately seconds, and as soon as the mixture is more homogeneous, take the second half of the powder and mix it until a total of 30 seconds have been completed (Rocha et al., 2003). On the Brazilian market there are also pre-dosed, encapsulated versions of the material available, to be mechanically manipulated in a capsule amalgamator. The cost of these materials is slightly higher, but they are fast and easy to manipulate and to insert the material into the cavity afterwards.

The material sensitivity to syneresis (dehydration) and imbibition (superhydration) in the first 24 hours after conventional glass ionomer cement has been inserted, results in loss of the mechanical properties of the material. To avoid these phenomena, it is imperative to protect the surface of the material, and light polymerizable adhesives. Copal cavity varnish, solid Vaseline or even nail varnish can be used. The longer this protective material is in contact with the restoration, the less chance there is of losing the mechanical properties of the material. The first 24 hours when the matrix is still being formed, is considered the critical period. When this phase has passed there is no longer any need for protection.

Due to syneresis and imbibition, finishing the restoration with diamond tips or water-cooled abrasive paper disks is also not indicated soon after the restoration has been made. The ideal is to remove the excesses with manual instruments, and after 24 hours, the final setting period, finishing and polishing can be done in the most convenient manner.

Other than its use as a restorative material, GICs are also indicated for another type of use as fossula and fissure sealant. There is sufficient evidence that GIC acts in a preventive manner against caries lesions on the occlusal surface, in the same manner as do the conventional resin sealants (Mickenautsch et al., 2008), in spite of having less mechanical retention.

4. THE DURABILITY OF THE BOND TO DENTIN

Clinically, different causes that act synergically contribute to the failure of restorations. The tooth-restorative material bond is subject to chemical, biological, mechanical and thermal degradation. Chemically hydrolysis is the main event as a result of the entry of water at the interface. Once at the interface, the water plays a preponderant role in degradation (Carrilho et al., 2005). Hydrolysis breaks down not only the bonds between the collagen fibers, but also those of the resinous polymers (Hashimoto et al., 2000b). This process can be accelerated by the action of enzymes released by bacteria, or from the dentin (Pashley et al., 2004).

As hydrolytic degradation only occurs in the presence of water, the hydrophilicity of the adhesive, its water sorption and the subsequent degradation are related (Tay et al., 2002a; Tay et al., 2003). This means that irrespective of the adhesive strategy (total acid etching or self-etching), the presence of hydrophilic monomers in the adhesive leads to the formation of hybrid layers that behave as permeable membranes that allow the flow of water, even after the adhesive has been polymerized (Tay et al., 2002b). These water diffusion pathways along the interface are visualized by electronic microscopy with the use of silver as tracer. This phenomenon was called **nanoleakage**, initially found by Sano et al. (1995), which occurs inside the hybrid layer, irrespective of the presence of marginal gap. Thus, the deposition of silver in specimens tested immediately (24 hours) represents residual water from the adhesive procedure, while in aged specimens, it represents water absorption and consequent degradation (Breschi et al., 2008). Adhesives with a higher percentage of hydrophilic monomers (simplified) exhibit a higher degree of permeability after polymerization, and consequently, a higher expression of nanoleakage (Tay et al., 2002b).

A systematic review of the literature on the clinical effectiveness of contemporary adhesive systems revealed that the three-step total acid etch adhesive systems and the two-step self-etching systems presented good and reliable clinical performance. Whereas, the two-step total acid etch systems were less favorable, while the one-step self-etching systems had an inefficient performance, demonstrating that simplification resulted in a loss of effectiveness (Peumans et al., 2005).

Since clinical studies demand time, cost and the real cause of failure cannot always be determined, laboratory studies are used to predict the behavior of materials, as there is a direct correlation between laboratory and clinical performance (De Munck et al., 2005).

In the laboratory, all classes of adhesive systems exhibit some morphological and mechanical evidence of degradation after three months of immersion in water. Thus the hydrolytic stability of polymerized adhesives is crucial. The best way to obtain this objective is by the application of a hydrophobic adhesive in a separate step, since the worst results in vivo and in vitro were obtained with two-step total acid etch adhesives (primer and adhesive united in one bottle) and one-step self-etching adhesives. Due to their hydrophilic nature, these adhesives attract water and degrade more quickly than hydrophobic adhesives. Therefore, the three-step adhesives continue to be the gold standard in terms of durability. It has been affirmed that any form of simplification in the adhesive protocol results in loss of effectiveness. But the two-step self-etch systems are also close to the gold standard and have the advantage of being less critical in terms of manipulation and reducing technique sensitivity (De Munck et al., 2005).

As far as durability of the GIC bond to dentin is concerned, De Munck et al. (2006) demonstrated that the bond strength values of a RMGIC were not affected after 12 months of storage in water with indirect exposure. Nevertheless, it was demonstrated that after four years of storage in water, with indirect exposure, the RMGIC suffered degradation (De Munck et al., 2004), suggesting that degradation occurs in the long term, but can be slower for the materials with a chemical bond mechanism.

5. STRATEGIES FOR IMPROVING THE PERFORMANCE OF ADHESIVE RESTORATIONS

In the search for a strong and lasting bond, the addition of functional agents to adhesive systems has been suggested. Adper Single Bond 2 contains the copolymers of polyalkenoic acid of the GICs developed by 3M ESPE, with the intention that they form chemical bonds with the calcium of the substrate (Osorio et al., 2002). Another adhesive system, Clearfil Protect Bond (Kuraray), contains a functional monomer (10-MDP) which provides chemical interaction with hydroxyapatite, whose precipitates come from the loss of calcium from the dentinal matrix and can contribute to the stability of the bond in the long term (Inoue et al., 2005). In this system, as with other materials, fluoride release can also prevent degradation of the interface by inhibiting enzymes, being responsible for its better behavior in the long term *in vitro* (Reis et al.,2007). Although the discovery of the action of metalloproteinases of the dentin matrix being provocative, the solution of 2% chlorhexidine, used after etching the dentin and before applying the adhesive, was shown to inhibit or decelerate *in vivo* degradation of the interface (Hebling et al., 2005; Carrilho et al., 2007). Although degradation had been observed at a subclinical level, no patient presented any sign or symptom of deterioration, such as pain or sensitivity on mastication during the follow-up period, and all the restorations were clinically acceptable, which suggests that degradation of the bond may take a long time to be noted in the clinical situation.

The glass ionomer cements (GICs) are capable of "self-adhesion" to the dental structure (Yoshida et al., 2000). Their action mechanism is based on two principles. The first is based on interdiffusion of the ionomeric components among the collagen fibers exposed by the polyacrylic acid, establishing micromechanical retention in accordance with the principle of hybridization (Van Meerbeek et al., 1992). The second principle is based on the ionic interaction between the carboxylic groups of the polyacrylic acid with the calcium ions of the hydroxyapatite, which remain bonded to the collagen fibers (Yoshida et al., 2000). The GICs were modified by the addition of resinous components that allowed them to bond to resin composite in order to be used as **glass ionomer adhesives.** An example of this type of system is Fuji Bond LC (GC, Japan). The glass ionomer adhesive is a diluted version of the resin modified GICs. The demineralizing action of the 20% polyacrylic acid results in the formation of a hybrid layer from 0.5 to 1 micrometer thick, and the remaining hydroxyapatite crystals establish ionic interaction with the carboxylic groups. It is not completely clear to what extent the chemical interaction contributed to the effectiveness of the bond, but it is estimated that it is favorable in terms of resistance to degradation, since it provides a close fit between the substrate and material (Van Meerbeek et al., 2003).

The final objective of adhesive procedures is the complete envelopment of collagen fibers by the bond agent, in order to protect the interface from degradation. Since the effectiveness and durability of the bond appear to depend on the quality of the hybrid layer, that is, complete impregnation of the substrate by the adhesive agent, strategies have been proposed for improving the infiltration of monomers, reducing the degree of water absorption and reducing the degradation of collagen, such as: 1) the use of systems in which the primer and the adhesive are separated; 2) increase in the polymerization time; 3) improved impregnation by means of a longer application time and friction, and 4) the use of MMP inhibitors (Breschi et al., 2008) such as chlorexidine before the adhesive, or so fluoride releasing materials.

6. BIOCOMPATIBILITY

Since dentin and pulp represent two phases of one and the same tissue, any procedure directed towards dentin will be received by the dentin-pulp complex. As dentin is mineralized, it presents a buffer action against acid etching, and this impedes the action of the acid as pulp irritant. In other words, a reaction occurs between the components of dentin, especially hydroxyapatite (alkaline) and the acid, limiting free diffusion of the acid along the tubules (Wang et al., 1988). The limited action of the acid on dentin can be verified by the thickness of the hybrid layer formed, between 1.9 and 5.8 micrometers (Perdigão, Lopes, 1999). The application of acid on dentin consists of a safe procedure, and a condition necessary for the sealant action of the present day adhesive systems. Nevertheless, in very deep cavities, with less than 300 micra (0.3 mm) of dentinal remainder, the thin dentinal barrier would not impede the passage of the acid and adhesive, resulting in problems with the pulp, such as a moderate inflammatory condition associated with tissue disorganization; and protection of the deepest part with a calcium hydroxide cement would then be indicated (Costa et al., 2002).

In the decade of the 1990s, some authors defended the direct application of acid and adhesive on the exposed pulp, attributing the success of the technique to the adequate sealing of the tooth/restoration interface and the correct diagnosis of the state of pulp health (Heitmann et al., 1995; Kanca et al., 1993). However, processes of pulp degeneration were demonstrated, both in dog and human teeth that received treatment with applications of acid and adhesive in deep cavities (300 micrometers of dentin remainder) and on exposed pulp (Costa et l., 2002; Costa et al., 2000). The pulp enters a process of early aging, due to the non-differentiation of mesenchymal cells into odontoblasts, responsible for the production of mineralized tissue (dentin bridges); followed by the necrotizing phase, in a slow, gradual manner, not observable in radiographs or in clinical reports. This pulp degeneration would be caused by the action of the acid on the surface and the diffusion of primer/adhesive monomers to within the pulp tissue, via the dentinal tubules (Tay et al., 1995). The fact that these substances are not digested by the macrophages creates a low pH environment favorable to cellular non-differentiation. The hydrostatic pressure of the pulp (25 mm Hg) is not sufficient to impede the diffusion of substances such as non-polymerized HEMA and TEGDMA (Gerzina et al., 1995). Furthermore, the monomers commonly present in adhesives have immunosuppressive capacity (Jontell et al., 1995).

In view of the polemic, it is believed that in view of the complete removal of carious tissue in deep cavities, protection of the pulp wall in its deepest portion with calcium hydroxide cement, leaving the lateral walls to the action of the acid and adhesive system, would be the most indicated clinical conduct. In the face of scientific, biological and clinical evidences that the partial removal of carious dentin, and maintaining the layer of affected dentin capable of being remineralized, prevents pulp exposure and later complications arising from pulp therapy, the clinical situation that uses calcium hydroxide cement seems to give way to hybridization over the dentin affected by caries.

7. ADHESIVE RESTORATION ON AFFECTED DENTIN

The current concepts of restorative Dentistry are characterized by the growing effort with regard to a less invasive approach to carious lesions. Only the softened and humid portion of the dentin is highly infected by bacteria, so that its removal is sufficient to assure paralyzation of the carious process, provided there is adequate sealing. The dentin remainder in these conservative preparations is affected, as it is demineralized, but it has viable odontoblastic processes and normal collagen capable of being remineralized (Nishitani et al., 2005; Massara et al., 2002). Therefore, the adhesive materials are being asked to adhere to different surfaces to those for which they were idealized, making it imperative to study adhesion to dentin affected by caries.

Affected intertubular dentin is more permeable than healthy intertubular dentin, due to the demineralization resulting from the carious process. The lowest hardness values found in affected dentin confirm that affected intertubular dentin is less mineralized in comparison with healthy dentin, in spite of the tubules being filled by minerals (Ceballos et al., 2003; Nakajima et al., 1999a; Nakajima et al., 1999b), which makes it more porous, favoring the creation of thicker hybrid layers (Nakajima et al., 1999b; Yoshiyama et al., 2002) by the facilitated diffusion of etching agents. Nevertheless, there is no correlation between the hybrid layer thickness and bond strength. This demonstrates that this greater thickness is due to a greater depth of demineralization of the affected and already demineralized dentin, which is not completely impregnated with adhesive, leaving a zone rich in exposed collagen, which affects the immediate bond strength and durability of the bond (Nakajima et al., 2005).

Investigations have demonstrated that for the majority of adhesive systems, in permanent teeth, the bond strength to dentin affected by caries is inferior to that to healthy dentin (Ceballos et al., 2003; Nakajima et al., 1999a; Yoshiyama et al., 2002; Erhardt et al., 2008). In primary teeth, using self-etching systems (Nakornchai et al., 2005; Hosoya et al., 2006) and RMGIC (Marquezan et al., 2008), no differences were found between healthy and affected dentin, which reinforces the hypothesis that the use of weaker acids prevents weakening of the already demineralized dentin. Thus the use of moderate self-etching adhesive systems and even glass ionomer-based systems that use polyacrylic acid, is encouraged on dentinal substrate affected by caries.

The activity of enzymes associated with degradation of the bond, the matrix metalloproteinases (MMPs), is greater in affected dentin (Hebling et al., 2005), and the interface with this substrate is more susceptible to degradation (Erhardt et al., 2008). Thus, it

is pertinent to consider the use of MMP inhibitors, such as **fluoride** and **chlorhexidine**, in an endeavor to decelerate degradation (De Munck et al., 2005).

In addition to the question of degradation, another aspect to consider as far as the durability of adhesive restorations is concerned, is the oral environment in which the adhesion is inserted. The clinical diagnosis of secondary caries has been referred to as the main reason for replacing restorations (Mjör et al., 2000). Furthermore, it has been demonstrated in vitro that cariogenic challenge significantly reduces the strength of adhesive restorations (Marquezan et al., 2008; Peris et al., 2007; Rocha et al., 2007), demonstrating that the loss of minerals at the interfaces in as a result of repeated falls in pH also open the way for restoration degradation. Vitremer, however, presented no compromise of bond strength after cariogenic challenge in vitro, which could be attributed to the chemical bond of the ionomeric material to the surrounding cavity walls (Marquezan et al., 2008).

The intrinsic fragility of dentin affected by caries should not be a clinical problem if there is healthy dentin and enamel present on the surrounding walls of the excavated lesion, promoting satisfactory bond strength values and durability. Furthermore, adequate sealing of the cavity guarantees paralyzation of the carious process and remineralization of the dentin remainder through viable odontoblastic processes (Massara et al., 2002, Falster et al., 2002). But sealing is the challenge, since the bond to the surrounding walls of the cavity is also subject to degradation in the long term (De Munck et al., 2006) and the cariogenic challenge is capable of reducing the bond strength values of the restoration even when there are surrounding walls of enamel and dentin present (Marquezan et al., 2008), emphasizing the need for control of the patient's carious activity to ensure the clinical success of minimally invasive restorations.

8. Sealing of Lesions: Non-Removal of Carious Tissue

The removal of carious tissue is one of the steps of restorative treatment, and there has been a great deal of discussion about the quantity of tissue to be removed and the type of remaining substrate for adhesion (Ricketts et al., 2006). The adhesion mechanism and its longevity in a substrate that differs from the one in which the materials were tested before they were released for sale is an extremely interesting field for research.

More recently, through the knowledge generated by researches on the mechanism of caries lesion formation and its arrest, a precedent was opened for not removing any carious tissue.

As previously mentioned, it is known that "the seal is the deal" and there are some researches that have suggested that instead of removing carious tissue from the lesion on the occlusal surface, and performing the bond on the carious dentin substrate, bond is performed at the expense of the enamel, sealing the caries lesion well, and avoiding the accumulation of biofilm on it. Thus the advance of the lesions is paralyzed, particularly those on the external half of the dentin, the main indication for the therapeutic sealing technique (Kramer et al., 2003; Hesse et al., 2007).

The therapeutic sealing of fossas and fissures with caries lesions has shown to be effective during the time in which the sealant remains intact on the surface, without loss of continuity (Elderton, 1985; Thylstrup and Fejerskov, 2001; Kidd, 2004). It is extremely

important for the bond to be adequate, but the use of adhesive systems before the application of sealants does not appear to improve the quality of the sealants (Hevinga et al., 2008), and is not indicated as a usual measure, since they demand more clinical time for application.

9. CONCLUSION

The decision to restore should form part of a treatment plan that involves control of the determinant factors of caries disease. This treatment plan must consider the needs related to the patient (age) and tooth (face, depth) in order to select the restorative material. Adhesive restorations, whether they are GIC or RC, although their clinical performance is recognized, are subject to subclinical degradation in the long term, and this degradation can be decelerated by the use of chlorhexidine or fluoride releasing materials. Control of carious activity in the patient is also determinant for the longevity of restorations.

REFERENCES

Berg JH. Glass ionomer cements. *Pediatr Dent* 2002; 24(5):430-38.

Bolaños-Carmona V, González-López S, De Haro-Muñoz C, Briones-Luján MT. Interfacial morphology and bond strength of self-etching adhesives to primary dentin with or without acid etching. *J Biomed Mater Res B Appl Biomater.* 2008 May 27. [Epub ahead of print]

Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano Dorigo E. Dental adhesion review: aging and stability of the bonded interface. *Dent Mater* 2008; 24(1):90-101.

Burrow MF, Nopnakeepong U, Phrukkanon S. A comparison of microtensile bond strengths of several dentin bonding systems to primary and permanent dentin. *Dent Mater* 2002; 18(3):239-45.

Burrow MF, Tyas MJ. 1-year climical eva¬lua¬tion of One-Step in non-carious cervical lesions. *Am J Dent* 1999; 12(6):283-5.

Carrilho MR, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, Reis AF, Hebling J, Mazzoni A, Breschi L, Pashley D. In vivo preservation of the hybrid layer by chlorhexidine. *J Dent Res.* 2007; 86(6):529-33.

Carrilho MRO, Tay FR, Pashley DH, Tjäderhane L, Carvalho RM. Mechanical stability of resin–dentin bond components. *Dent Mater* 2005; 21:232-41.

Carvalho RM, Yoshiwama M, Pashley EL, Pashley DH. In vitro study on the dimensional changes of human dentine after demineralization. *Arch Oral Biol* 1996; 41:369-77.

Casagrande L, de Hipólito V, de Góes MF, Barata JS, Garcia-Godoy F, de Araújo FB. Bond strength and failure patterns of adhesive restorations in primary teeth aged in the oral environment. *Am J Den*t 2006; 19(5):279-82.

Ceballos L, Camejo DG, Victoria Fuentes M, Osorio R, Toledano M, Carvalho RM, Pashley DH. Microtensile bond strength of total-etch and self-etching adhesives to caries-affected dentine *J Dent* 2003; 31(7):469-77.

Costa CAS, Hebling J, Hanks CT. Curentes status of pulp capping with dentin adhesive systems: a review. *Dent Mater* 2000; 16(3):188-97.

Costa, C.A.S.; Nascimento, A.B.L.; Teixeira, H.M. – Response of human pulps following acid conditioning and application of a bonding agent in deep cavities. *Dent Mater* 2002; 18(7):543-51.

De Munck J, Shirai K, Yoshida Y, Inoue S, Van Landuyt K, Lambrechts P, Suzuki K, Shintani H, Van Meerbeek B. Effect of water storage on the bonding effectiveness of 6 adhesives to Class I cavity dentin. *Oper Dent* 2006;31(4):456-65.

De Munck J, Van Landuyt K, Peumans M, Poitevin A, Lambrechts P, Braem M, Van Meerbeek B. A critical review of the durability of adhesion to tooth tissue: methods and results. *J Dent Res* 2005; 84(2):118-32.

De Munck J, Van Meerbeek B, Yoshida Y, Inoue S, Suzuki K, Lambrechts P. Four-year water degradation of a resin-modified glass-ionomer adhesive bonded to dentin. *Eur J Oral Sci* 2004;112(1): 73–83.

Elderton RJ. Management of early dental caries in fissures with fissures sealant. *Br Dent J.* 1985;158(7):254-8.

Erhardt MC, Rodrigues JA, Valentino TA, Ritter AV, Pimenta LA. In vitro microTBS of one-bottle adhesive systems: sound versus artificially-created caries-affected dentin. *J Biomed Mater Res B Appl Biomater.* 2008; 86(1):181-7.

Falster CA, Araujo FB, Straffon LH, Nör JE. Indirect pulp treatment: in vivo outcomes of an adhesive resin system vs calcium hydroxide for protection of the dentin-pulp complex. *Pediatr Dent* 2002; 24(3):241-8.

Frankenberger R, Perdigão J, Rosa BT, Lopes M. 'No-bottle' vs. 'multi-bottle' dentin adhe¬sives - a microtensile strength and morphological study. *Dent Mater* 2001; 17(5):373-80.

Gerzina, T.M.; Hume, W.R. – Effect of hydrostatic pressure on the diffusion of monomers through dentin, in vitro. *J Dent Res* 1995; 74(1):369-73.

Goes MF, Pachane CGF, Garcia-Godoy F. Resin bond strenght with different methods to remove excess water from the dentin**.** *Am J Dent* 1997; 10(6):298-301.

Gwinnett AJ. Chemically conditioned dentin: A comparison of conventional and environmental scanning electron microscopy findings. Dent Mater 1994; 10(3):150-5.

Gwinnett AJ. Dentin bond strength after air drying and rewerting. *Am J Dent* 1994; 7(3):144-8.

Hashimoto M, Ohno H, Endo K, Kaga M, Sano H, Oguchi H. The effect of hybrid layer thickness on bond strength: demineralized dentin zone of the hybrid layer. *Dent Mater* 2000a; 16(6):406-11.

Hashimoto M, Ohno H, Kaga M, Endo K, Sano H, Oguchi H. In vivo degradation of resin-dentin bonds in humans over 1 to 3. *J Dent Res* 2000b; 79(6):1385-91.

Hebling J, Pashley DH, Tjaderhane L, Tay FR. Chlorhexidine Arrests Subclinical Degradation of Dentin Hybrid Layers in vivo. *J Dent Res* 2005; 84(8):741-6.

Heitmann T, Unterbrink G. Direct pulp capping with a dentinal adhesive system: a pilot study. *Quintessence Int* 1995; 26(1):765-70.

Hesse D, Bonifácio CC, Raggio DP, Imparato JCP. Avaliação do selamento de lesões de cárie comparado à restauração com resina composta em dentes decíduos. *Stomatos*. 2007;13(25):75-85.

Hevinga MA, Opdam NJ, Frencken JE, Bronkhorst EM, Truin GJ. Can caries fissures be sealed as adequately as sound fissures? *J Dent Res*. 2008;87(5):495-8.

Hosoya Y, Kawada E, Ushigome T, Oda Y, Garcia-Godoy F. Micro-tensile bond strength of sound and caries-affected primary tooth dentin measured with original designed jig. *J Biomed Mater Res B Appl Biomater* 2006; 77(2):241-8.

Inoue H, Inoue S, Uno S, Takahashi A, Koase K, Sano H. Microtensile bond strenght of two single-step adhesive systems to bur-pre¬pa¬red dentin. J Adhes Dent 2001; 3(2):129-35.

Inoue S, Koshiro K, Yoshida Y, De Munck J, Nagakane K, Suzuki K, et al. Hydrolytic stability of self-etch adhesives bonded to dentin. *J Dent Res* 2005; 84(12):1160–4.

Jontell M, Hanks CT, Bratel J, Berge¬nholtz G. Effect of unpolymerized resin com¬po-nentes on the function of accessory alls de¬rived from the rat incisor pulp. *J Dent Res* 1995; 74(5):1162-67.

Kanca J. Replacement of a fractured incison fragment over pulpal exposure: a case report. *Quintessence Int* 1993; 24(2):81-4.

Kidd EA. How "clean" must be a cavity before restoration? Caries Res. 2004;38(3):305-13.

Krammer PF, Cardoso L, Reis ASP, Silveira D, Tovo MF. Efeito da aplicação de selantes de fossas e fissuras na progressão de lesões cariosas oclusais em dentes decíduos: observações clínicas e radiográficas. *Rev Ibero-am Odontopediatr Odontol Bebê.* 2003;6(34):504-14.

Leinfelder KF, Kurdziolek S. Glass ionomers revisited. *Dent Today* 2001; 20(1):36-43.

Marquezan M, Osorio R, Ciamponi AL, Toledano M. Resistance to degradation of bonded restorations to caries-affected primary dentin. *Am J Dent.* In press 2008.

Massara ML, Alves JB, Brandão PR. Atraumatic restorative treatment: clinical, ultrastructural and chemical analysis. *Caries Res* 2002; 36(6):430-6.

Mickenautsch S, Yengopal V, Bönecker M et al. *Minimal Intervention: a new approach in dentistry.* 3. ed. 2008. v. 1. 135p.

Miyazaki M, Iwasaki K, Onose H, Moo¬re BK. Enamel and dentin bond strenghts of sin-gle application bonding systems. *Am J Dent* 2001; 14(6):361-6.

Miyazaki M, Onose H, Moore BK. Effect of operator variability on edentin bond strength of two-step bondingsystems. *Am J Dent* 2000; 13(2):101-4.

Mjör IA, Moorhead JE, Dahl JE. Reasons for replacement of restorations in permanent teeth in general dental practice. *Int Dent J* 2000; 50(6):361-6.

Mount GJ. *An atlas of glass ionomer cement: a clinician's guide.* London:Martin Dunitz 1994. 150 p. 2nd ed.

Nakabayashi N, Ashizawa M, Nakamura M. Identification of a resin-dentins hybrid layer in vital human dentin created in vivo: durable bonding to vital dentin. *Quintessence Int* 1992; 23(2):135-41.

Nakabayashi N, Kojima K, Mashuara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. *J Biomed Mat Res* 1982; 16(3):265–73.

Nakajima M, Kitasako Y, Okuda M, Foxton RM, Tagami J. Elemental distributions and microtensile bond strength of the adhesive interface to normal and caries-affected dentin. *J Biomed Mater Res B Appl Biomater* 2005; 72(2):268-75.

Nakajima M, Ogata M, Okuda M, Tagami J, Sano H, Pashley DH. Bonding to caries-affected dentin using self-etching primers. *Am J Dent* 1999a; 12(6):309-14.

Nakajima M, Sano H, Zheng L, Tagami J, Pashley DH. Effect of moist vs. dry bonding to normal vs. caries-affected dentin with Scotchbond Multi-Purpose Plus. *J Dent Res* 1999b; 78(7):1298-303.

Nakornchai S, Harnirattisai C, Surarit R, Thiradilok S. Microtensile bond strength of a total-etching versus self-etching adhesive to caries-affected and intact dentin in primary teeth. *J Am Dent Assoc* 2005; 136(4):477-83.

Navarro MFL, Pascotto RC. *Cimentos de Ionômero de Vidro. São Paulo:* Artes Médicas: Série EAP-APCD, 1998. 179p.

Nishitani Y, Yoshiyama M, Tay FR, Wadgaonkar B, Waller J, Agee K, et al. Tensile strength of mineralized/demineralized human normal and carious dentin. *J Dent Res* 2005; 84(11):1075-8.

Nör JE, Feigal RJ, Dennison JB, Edwards CA. Dentin bonding: SEM comparison of the resin-dentin interface in primary and permanent teeth. *J Dent Res* 1996;75(6):1396-403.

Nör JE, Feigal RJ, Dennison JB, Edwards CA. Dentin bonding: SEM comparison of the dentin surface in primary and permanent teeth. *Pediatr Dent* 1997; 19(4):246-52.

Osorio R, Ceballos L, Tay F, Cabrerizo-Vilchez MA, Toledano M. Effect of sodium hypochlorite on dentin bonding with a polyalkenoic acid-containing adhesive system. *J Biomed Mater Res* 2002; 60(2):316-24.

Pashley DH, Ciucchi B, Sano H, Horner JA. Permeability of dentin to adhesive agents. *Quintessence Int* 1993; 24(9):618-31.

Pashley DH, Pashley EL, Carvalho RM, Tay FR. *The effects of dentin permeability on the restorative dentistry*. Den Clin North Am 2002; 46(2): 211-45.

Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al.Collagen degradation by host-derived enzymes during aging. *J Dent Res* 2004; 83(3):216-21.

Pashley DH, Zhang Y, Agee KA, Rouse CJ, Carvalho RM, Russell CM. Permea¬bi¬lity of demineralized dentin to HEMA. *Dent Mater* 2000; 7(1):7-14.

Perdigão J, Carmo ARP, Geraldeli HR, Masuda MS. Six month clinical evaluation of two dentin adhesiver applied an dry vs. Moist dentin. *J Adhes Dent* 2001; 4(4):343-52.

Perdigão J, Frankenberger R. Effect of solvent and rewetting time on dentin adhesion. *Quintessence Int* 2001; 32(5):385-90.

Perdigão J, Lambrechts P, Van Meerbeek B, Vanherle G, Lopes LB. Field emission SEM comparision of four postfixation drying tech¬ni¬ques for human dentin. *J Biomed Mater Res* 1995; 29(9):1111-20.

Perdigão J, Lopes M. *Dentin Bonding* - State of the art 1999. Compendium 1999; 20(12):1151-62.

Perdigão J, Van Meerbeek B, Lopes MM, Ambrose WW. The effect of a re-wetting agent on dentin bondin. *Dent Mat* 1999; 15(4):282-95.

Peris AR, Mitsui FH, Lobo MM, Bedran-Russo AK, Marchi GM. Adhesive systems and secondary caries formation: Assessment of dentin bond strength, caries lesions depth and fluoride release. *Dent Mater* 2007; 23(3):308-16.

Peumans M, Kanumilli P, De Munck J, Van Landuyt K, Lambrechts P, Van Meerbeek B. Clinical effectiveness of contemporary adhesives: a systematic review of current clinical trials. *Dent Mater* 2005; 21(9):864-81.

Reis AF, Giannini M, Pereira PN. Long-term TEM analysis of the nanoleakage patterns in resin-dentin interfaces produced by different bonding strategies. *Dent Mater* 2007; 23(9):1164-72.

Ricketts DN, Kidd EA, Innes N, Clarkson J. *Complete or ultraconservative removal of decayed tissue in unfilled teeth.* Cochrane Database Syst Rev. 2006.

Rocha RO, Oliveira LB, Raggio DP, Rodrigues CRMD. Cimento de ionômero de vidro como selantes de fossas e fissuras. *Revista da APCD*, 2003;57(4):287-90.

Rocha RO, Soares FZMS, Rodrigues Filho LE, Rodrigues CRMD. Influence of aging treatments on microtensile bond strength of adhesive systems to primary dentin. *J Dent Child* 2007; 74(2):109-112.

Sano H, Takatsu T, Ciucchi B, Horner JA, Matthews WG, Pashley DH. Nanoleakage: leakage within the hybrid layer. *Oper Dent* 1995; 20(1):18-25.

Sardella TN, de Castro FL, Sanabe ME, Hebling J. Shortening of primary dentin etching time and its implication on bond strength. *J Dent* 2005; 33(5):355-62.

Shono Y, Ogawa T, Terashita M, Carvalho RM, Pashley EL, Pashley DH. Regional Measurement of Resin-Dentin Bonding as an Array. *J Dent Res* 1999; 78(2): 699-705.

Tay FR, Gwinnett AJ, Pang KM, Wei SHY. Micromorphologic relationship of the resin-dentin interface following a total-etch technique in vivo using a dentinal bondind system. *Quintessence Int* 1995; 26(1):63-70.

Tay FR, Gwinnett AJ, Wei SHY. The overwet phenomenon: a scanning electron microscopic study of surface moisture in the acid-conditioned, resin-interface. *Am J Dent* 1996; 9(3):109-14.

Tay FR, Gwinnett AJ, Wei SHY. Ultras¬truc¬ture of the resin-dentin interface following reversible and irreversible rewetting. *Am J Dent* 1997; 10(2):77-82.

Tay FR, Hashimoto M, Pashley DH, Peters MC, Lai SC, Yiu CK, et al. Aging affects two modes of nanoleakage expression in bonded dentin. *J Dent Res* 2003; 82(7):537-41.

Tay FR, Pashley DH, Suh BI, Carvalho RM, Itthagarun A. Single-step adhesives are permeable membranes. *J Dent* 2002b; 30(7-8):371-82.

Tay FR, Pashley DH, Yoshiyama M. Two modes of nanoleakage expression in single-step adhesives. *J Dent Res* 2002a; 81(7):472-6.

Tay, F.R.; Pashley, D.H. Aggessives of contemporary self-etching systems. I: Depth of penetration beyong dentin smear layers. *Dent Mater* 2001; 17(4):296-308.

Thylstrup A, Fejerskov O. Cariologia Clínica. 3ed. São Paulo:Santos, 2001.

Toledano M, Osorio R, Leonardi G, Leal JIR, Ceballos L, Vilchez MAC. Influence of self-etching primer on the resin adhesion to enamel and dentin. *Am J Dent* 2001; 14(4):205-10.

Uekusa S, Yamaguchi K, Miyazaki M, Tsubota K, Kurokawa H, Hosoya Y. Bonding efficacy of single-step self-etch systems to sound primary and permanent tooth dentin. *Oper Dent* 2006; 31(5):569-76.

Van Meerbeek B, De Munck J, Yoshida Y, Inoue S, Vargas M, Vijay P, et al. Buonocore memorial lecture. Adhesion to enamel and dentin: current status and future challenges. *Oper Dent* 2003; 28(3):215-35.

Van Meerbeek B, Inokoshi S, Braem M, Lambrechts P, Vanherle G. Morphological aspects of the resin-dentin interdiffusion zone with different dentin adhesive systems. *J Dent Res* 1992; 71(8):1530-40.

Van Meerbeek B, Vargas M, Inoue Yoshida Y, Peumans M, Lambrechts P, Vanherle G. Adhesives and cements to promote preservation dentistry. *Oper Dent* 2001; 6(6): 119-44.

Wang, J.D.; Hume, W.R. – Diffusion of hydrogen ion and hydroxyl ion from various sources through dentine. *Int Endod J,* v.21, n.1, p.17-26, Jan. 1988.

Wilson AD, Kent BE. A new translucent cement for dentistry. The glass ionomer cement. *Br Dent J* 1972 15;132(4):133-5.

Yoshida Y, Van Meerbeek B, Nakayama Y, Snauwaert J, Hellemans L, Lambrechts P, Vanherle G, Wakasa K. Evidence of chemical bonding at biomaterial-hard tissue interfaces. *J Dent Res* 2000; 79(2):709-14.

Yoshiyama M, Tay FR, Doi J, Nishitani Y, Yamada T, Itou K, et al. Bonding of self-etch and total-etch adhesives to carious dentin. *J Dent Res* 2002; 81(8):556-60.

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Chapter 5

EFFECTS OF METHACRYLATES PRESENT IN DENTAL COMPOSITE RESINS ON HL-60 CELL METABOLISM

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ABSTRACT

Composite resins were introduced in the 1960s for the restoring of anterior teeth in substitution of the amalgam that presented both aesthetical and biocompatibility problems.

However, since the polymerization of methacrylates is never complete, it became evident the necessity to appraise the biocompatibility of composite resins. The incomplete conversion causes in fact the release of monomers that may implement adverse effects in the organism, i.e., allergic reactions, systemic toxicity, cytotoxicity, estrogenicity and mutagenicity. Because very little information has been so far delivered on the consequences of methacrylic monomers on cell metabolism, we were driven to investigate the biochemical interactions between methacrylates and human cells. The present work summarizes the effects of TEGDMA, UDMA, BDDMA, HEMA and Bis-GMA on 1) cellular energetic metabolism (oxygen consumption rate, glucose consumption, G6PDH, lactate production) and 2) cellular redox status (GSH concentration, and the activity of the enzymes regulating glutathione metabolism). The results obtained showed that all monomers induced both cellular differentiation and a decrease of oxygen consumption. Moreover, cells treated with TEGDMA and HEMA showed a significant enhancement of glucose consumption and lactate production, induced GSH depletion and stimulated G6PDH and GR activity. BDDMA also induced GSH depletion but without any effect on the activity of the enzymes involved in

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glutathione metabolism, while UDMA did not change GSH content and redox metabolism at all.

The mechanism of the differentiating action can be basically reconducted to an impairment of the mitochondrial respiration which starts two hours after incubation of the cells with each monomer and determines in the following hours an increase of anaerobic glycolysis. Thus the changes in energy metabolism and glutathione redox balance could be considered a potential mechanism for inducing clinical and sub-clinical adverse effects and thus providing a wider look for testing biocompatibility of dental materials.

1. INTRODUCTION

Composite resins utilized in medicine—largely in dental but also in orthopaedic field [1,2]—are complex mixed material which consist of an organic polymerizable matrix and an inorganic reinforcing filler coupled by a silanic agent [3,4]. Resinous matrix is frequently composed by Bis-phenol A glycerolate dimethacrylate (Bis-GMA) with addition of other methacrylic monomers whose main function is to improving the handling and get an easier incorporation of the filler; due to their lower viscosity, the most used compounds are triethylenglycol-dimethacrylate (TEGDMA), 2-hydroxyhethyl methacrylate (HEMA), urethane-dimethacrylate (UDMA) and occasionally 1,4-butanediol dimethacrylate (BDDMA).

After performing dental restorations with composite resins, small amounts of monomers are released into either the oral cavity and—through dentinal diffusion [5-7]—in pulpal tissues, hence leaching into blood circulation [5]. The phenomenon may be due both to the presence of unpolymerized molecules since the reaction is never complete, and to other compounds (including monomers) leached through erosion and chemical degradation caused by hydrolysis catalyzed by unspecific and human saliva-derived esterases [8]. Subsequently, the monomers may cause, or at least contribute to, adverse biological effects [9], i.e., damage to the oral soft tissues already observed in vivo [10], and show a remarkable cytotoxic potential as observed in in vitro studies in primary and immortalized cultures [5,6,11].

In particular, in vitro studies carried out utilizing TEGDMA, Bis-GMA and HEMA, have underlined that these monomers have genotossic, allergenic [12], cytotoxic, estrogenetic (chiefly for Bis-GMA and TEGDMA) and mutagenic activity. Moreover these monomers alter lipid metabolism, glutathione (GSH) concentration, reactive oxygen species (ROS) production, cell cycle, energy metabolism and mitochondrial activity [13-19]. TEGDMA, furthermore, suppresses heat shock protein 72 expression in human monocytes [20], regulates glutathione transferase P1 [21] while HEMA reduces intracellular tyrosine phosphorylation [22].

Especially intringuing for us was TEGDMA effect on the mitochondria and consequent ROS production in human fibroblasts [16] and similarly interesting was HEMA capacity to increase ROS production [18] by an unknown mechanism.

Hence we postulated that also other methacrylic monomers could be able to induce mitochondrial damage and we have verified whether Bis-GMA, TEGDMA, BDDMA, UDMA and HEMA monomers were able to alter both energy metabolism and the enzymes regulating the GSH balance. Energy metabolism was investigated by assessing a) oxygen and glucose consumption and b) lactate production through anaerobic glycolysis. Cell

differentiation, which can result from metabolic alterations due to mitochondrial dysfunction, was also monitored [23]. Moreover, GSH redox metabolism was investigated through the assay of glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) activity, the former being the NADPH-dependent enzyme catalyzing GSSG reduction and the latter being the enzyme regulating the rate of the hexose monophosphate (HMP) shunt [24], i.e., the metabolic pathway producing NADPH [25] from glucose. Cells can synthesize GSH also starting from amino acids with consequent energy consumption, as indicated by an increase of the [diphosphate nucleoside] / [triphosphate nucleoside] ratio [26].

The well-characterized promyelocytic HL-60 cell line was employed in our studies as a suitable experimental model for its sensitivity to cytotoxic, metabolic and differentiating agents [27]—including dental materials [28]—as well as for the recovery of the respiratory burst [29] after differentiation.

2. MATERIALS AND METHODS

All chemicals and reagents were obtained from Sigma-Aldrich Srl, Milan, Italy, unless otherwise indicated. Human leukemic HL-60 cell line (Istituto Zooprofilattico, Brescia, Italy) was maintained under a CO_2 humidified atmosphere (5%, 37°C) in RPMI 1640 with heat inactivated Fetal Calf Serum (10% v/v), penicillin (100 units/mL), streptomycin (100.0 μg/mL) and glutamine (2.00 mmol/L).

2.1. Preparation of All-*trans* Retinoic Acid and Methacrylates Solutions

Stocked dimethyl sulfoxide (DMSO) solutions of TEGDMA (from 3.10 mmol/L to 3.50 x 10^3 mmol/L), Bis-GMA (from 8.00 x 10^{-3} mmol/L to 0.12 mmol/L), UDMA (from 27.5 mmol/L to 220.0 mmol/L), BDDMA (0.2 mol/L and 0.4 mol/L) and All-*trans* Retinoic Acid (ATRA, 1.00 mmol/L) were prepared immediately before use. As a general procedure, one of the above described solutions (1.0 μL) was added to HL-60 cells (200,000) in RPMI 1640 medium (1.0 mL); whereas HEMA was added to the cells in a pure form to reach a final concentration ranging from 0.11 mmol/L to 1.10 mmol/L. DMSO (final concentration 0.1 % v/v) was utilized in all samples except in HEMA-treated cells and preliminary studies were performed with regard to DMSO effect on HL-60 cells.

2.2 Cell Viability

Exponentially growing HL-60 cells (15 x 10^6) in RPMI 1640 (75.0 mL) were incubated with either ATRA (1.00 x 10^{-3} mmol/L) or HEMA (0.11 and 1.1 mmol/L) or Bis-GMA (0.008 and 0.16 mmol/L) or TEGDMA (from 0.03 to 3.1 mmol/L) or UDMA (from 27.5 x 10^{-3} mmol/L to 220.0 x 10^{-3} mmol/L) or BDDMA (0.2 mmol/L and 0.4 mmol/L) for five days. The total cell number was determined every day using the trypan blue dye exclusion test. Both the cellular proliferation and cellular mortality—the latter expressed as area under curves (AUC)—were calculated.

2.3 Assays Condition

All the experiments described below were performed with either ATRA (1.00×10^{-3} mmol/L), HEMA (1.10 mmol/L), TEGDMA (0.38 mmol/L) or Bis-GMA (1.60×10^{-2} mmol/L) or UDMA (27.5×10^{-3} mmol/L and 55.0×10^{-3} mmol/L) or BDDMA (0.2 mmmol/L and 0.4 mmol/L) and the incubations were performed in a humidified atmosphere of 5% CO_2.

2.4. Differentiation Assay

The respiratory burst of methacrylate-treated cells was assessed through chemiluminescence technique (CL) [30]. Both untreated and treated HL-60 cells (1×10^5), either unstimulated or stimulated with phorbol 12-myristate 13-acetate (1.5 nmol), were treated with luminol (100.0 nmoles) to prepare CL specimens. All samples were adjusted to final volume (1.0 mL) by adding Krebs-Ringer-phosphate (KRP) buffer solution and measurements were performed using an automatic luminometer (Autolumat LB 953, EG&G, Turku, Finland) for 120 min at 25 °C and results were expressed as signal (corrected by subtracting background luminescence) of treated *vs* untreated cells.

2.5. Oxygen Consumption Rate

Exponentially growing HL-60 cells (20×10^6) in RPMI 1640 (100.0 mL) were incubated with ATRA or UDMA (55.0×10^{-3} mmol/L) or BDDMA (0.4 mmol/L) (1 h at room temperature), washed (PBS solution with neither Ca^{++} nor Mg^{++}), resuspended in KRP buffer (10×10^6 cells/mL) and finally utilized to monitor the oxygen consumption rate under constant stirring, 10 min at room temperature (Oxygen meter Model 781, Strathkelvin Instruments, Glasgow, UK).

2.6. Determination of Cellular Glucose Consumption

Exponentially growing HL-60 cells (1×10^6) in RPMI 1640 (5.0 mL) were incubated with either ATRA or one of the monomers (24 h and 48 h, 37 °C). Cellular glucose consumption was measured in the culture supernatants (Hitachi 917 automatic analyzer, Roche Diagnostics AG, Basel, Switzerland) with the appropriate reagent kit (Glucose GOD-PAP-HK, Roche Diagnostics AG). In order to normalize glucose consumption at different cell proliferation rates, data were expressed as the ratio:

$$\frac{\text{Glucose concentration (mg/mL, 24 h) - Glucose concentration (mg/mL, 48 h)}}{\text{AUC of grown cells (from 24 h to 48 h)}}$$

2.7. Determination of Cellular Lactate Production

Lactate concentration changes were evaluated on culture medium by ^1H nuclear magnetic resonance analysis [27] (NMR Gemini 300 spectrometer, Varian, Palo Alto, CA). HL-60 exponentially growing cells (1.2 x 10^6) in RPMI 1640 (6.0 mL) were incubated with either ATRA or one of the monomers (48h) and, after cells removal, medium (3.0 mL) was mixed with ice cold $HClO_4$ (25 %, 1.0 mL) and centrifuged (3800 *g*, 20 min). The pellets were discarded and the solution was neutralized with K_2CO_3, lyophilized and then dissolved in H_2O (0.5 mL) + D_2O (0.2 mL) with 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP, 0.21%,). Results were calculateded as follows:

$$\frac{\text{\% lactate signal at 1.33 ppm } vs \text{ TSP signal of treated cells / AUC}}{\text{\% lactate signal at 1.33 ppm } vs \text{ TSP signal of untreated cells / AUC}} \times 100$$

2.8. Determination of G6PDH and GR Enzymatic Activities

Exponentially growing HL-60 cells (10 x 10^6) in RPMI 1640 (50.0 mL) were incubated with ATRA or one of the monomers (24 h or 48 h). The enzymatic activities were then determined in cell extracts: cellular pellets obtained after centrifugation (400 *g*, 5 min, 4 °C) were washed in PBS solution and stored (-80 °C, 7 days). Cell lysates were centrifuged (20,000 *g*, 15 min, 4 °C) and the supernatants collected were used to determine the protein content (BioRad Protein Assay); the enzymatic activities were measured (Olympus AU 400 Chemistry Analyzer) with the appropriate G6PDH and GR Randox kits (Randox kit; Randox Laboratories Ltd,. Ardmore, UK).

2.9. Cellular Glutathione Determination

Exponentially growing HL-60 cells (10 x 10^6) in RPMI 1640 (50.0 mL) were incubated with either ATRA or one of the monomers (1 h, room temperature); the cells were then washed twice with PBS, resuspended in trichloroacetic acid (6 %, 100.0 µL) and immediately stirred. The cells lysed were centrifuged (20,000 *g*, 4 min) and the supernatants were used to establish GSH and GSH + GSSG (total glutathione) quantity. GSH concentration was determined by Ellman method [31], modified by Wataha [32]: the supernatant (40.0 µL) was added to Na_2HPO_4 (0.30 mol/L, 80 µL) and 5,5'-Dithiobis[2-nitrobenzoic acid] (DTNB, 0.04 % in 1 % sodium citrate, 10.0 µL). Absorbance was measured at 405 nm (Packard Spectracount™, Packard BioScience Company, Meriden CT U.S.A.) reporting the results as the percentage of treated *vs* untreated cells. The total glutathione was estimated as follows: a freshly prepared $NaBH_4$ aqueous solution (20.00 mg/mL, 40.0 µL) was added to the supernatant (40.0 µL) previously shaken with ethyl ether (120.0 µL) to remove the lypophilic substances. After incubation of the mixture (40 min, 37 °C), HCl (1 N, 37.5 µL), acetone (40.0 µL) and Tris buffer (1.0 mol/L, pH 8.5, 30.0 µL) were added and an aliquot of the solution (150.0 µL) was mixed with DTNB (0.04 % in 1.0 % sodium citrate, 10.0 µL) to determine its concentration spectrophotometrically, as above reported.

2.10. Statistical Analysis

Data are expressed as the mean ± statistical error of the mean (SEM) of at least three different experiments performed in duplicates. The means were compared by analysis of variance (ANOVA) followed, when appropriate, by a multiple comparison of means by Student-Newman-Keuls test: $p < 0.05$ was considered significant.

3. RESULTS

3.1. HEMA

This monomer did not induce any effect on cellular proliferation and cell mortality at the concentrations here employed (Fig. 1) and acted as a cellular differentiating agent at a concentration of 1.1 mmol/L ($p< 0.011$) (Fig. 2).

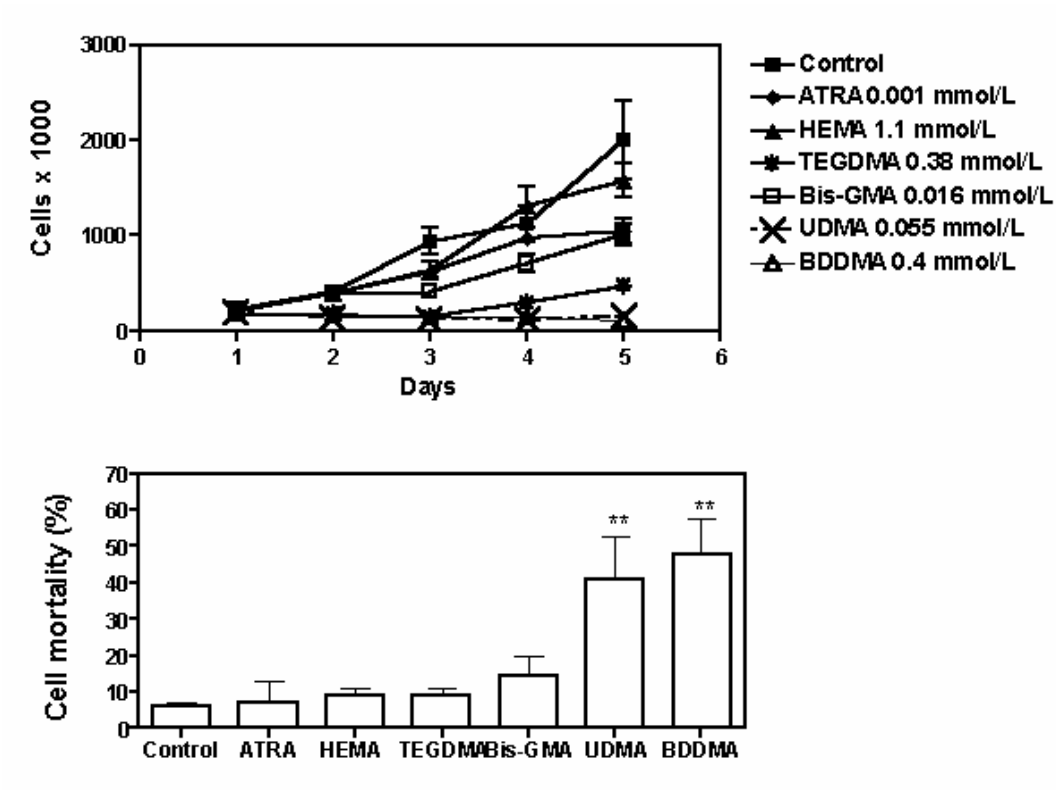

Figure 1. Proliferation curves and mortality of HL-60 cells: untreated or treated with ATRA or methacrylates. Total cell number was determined every day using the trypan blue dye exclusion test; ** ($p<0.01$) significantly different from control. Further details in Materials and Methods.

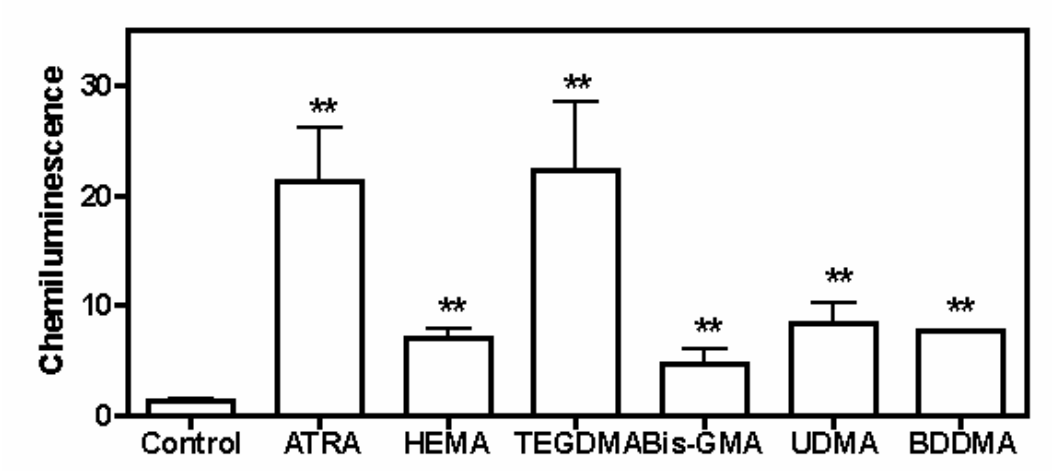

Figure 2. Chemiluminescence analysis of HL-60 cells: untreated and treated with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L). The cells were incubated for 5 days and measurements were performed for 120 min at 25 °C. The results were expressed as the signal (corrected by subtracting background luminescence) of PMA-stimulated vs unstimulated cells; ** ($p<0.01$) significantly different from control. Further details in Materials and Methods.

HEMA caused a decrease of oxygen consumption rate in HL-60 cells ($p<0.05$) (Fig.3), while glucose intake and lactate production remain unaffected (Figs. 4 and 5 respectively); G6PDH and GR activity increased ($p<0.01$) (Fig. 6) and a decrease of GSH and total glutathione is observed ($p<0.01$) (fig. 7).

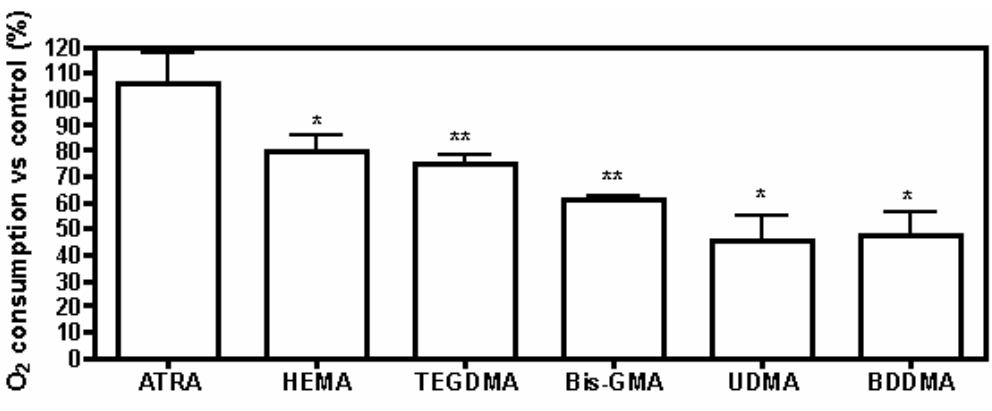

Figure 3. Oxygen consumption rate in HL-60 cells: after treatment with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L). The results were reported as percentage of oxygen consumption rate of treated cells vs control; *($p<0.05$) and ** ($p<0.01$) significantly different from ATRA. Further details in Materials and Methods.

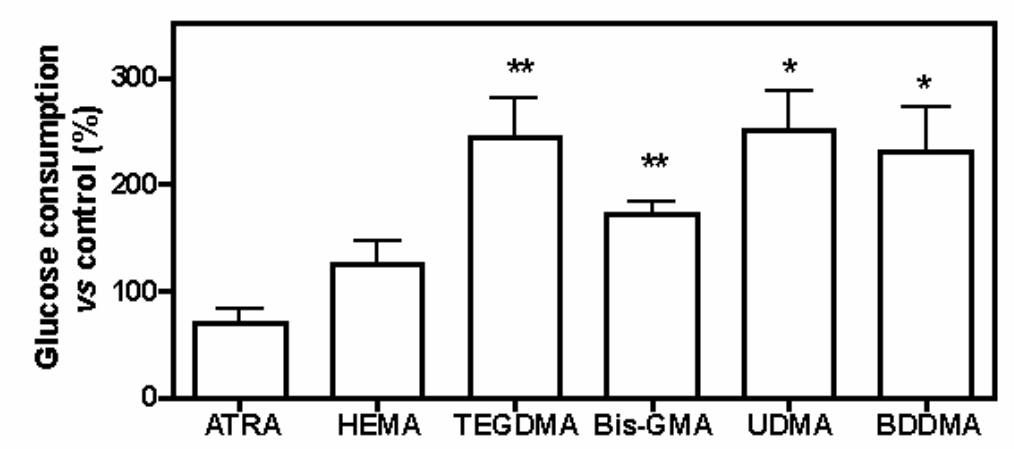

Figure 4. Glucose disposal in HL-60 cells: untreated and treated with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L). The results were reported as percentage of glucose consumption of treated cells vs control; *($p<0.05$) and ** ($p<0.01$) significantly different from ATRA. Further details in Materials and Methods.

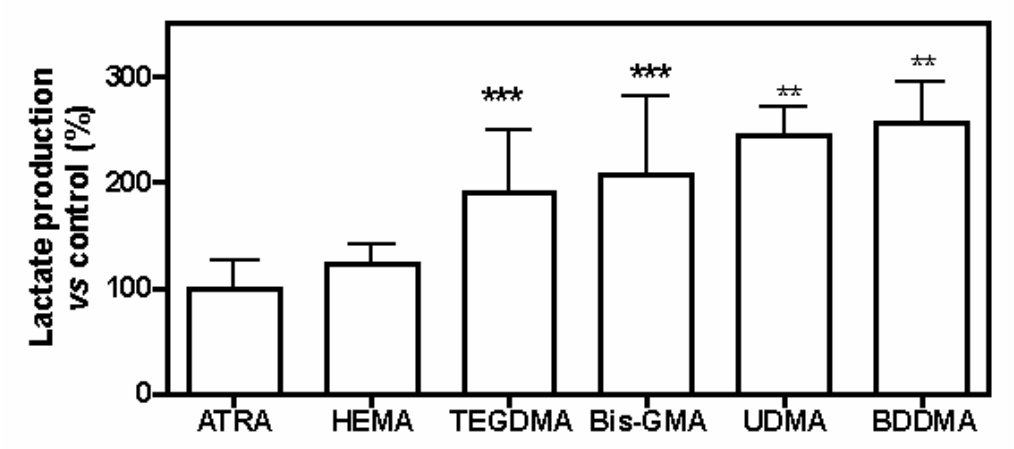

Figure 5. Lactate production by HL-60 cells: untreated and treated with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L). The results were reported as percentage of lactate produced by treated cells vs control; ** ($p<0.01$) and *** ($p<0.001$) significantly different from ATRA. Further details in Materials and Methods.

3.2. TEGDMA

This monomer induced a significant decrease in the cellular proliferation rate at concentrations higher than 0.09 mmol/L and a significant cellular mortality at concentrations above 0.58 mmol/L (Fig. 1). TEGDMA was utilized for all experiments on cell metabolism at a concentration of 0.38 mmol/L because this value elicited the greatest decrease in cellular proliferation ($p<0.01$) (cytostatic effect) without increasing cell mortality.

As reported for HEMA, also TEGDMA caused in HL-60 cells: differentiation ($p<0.01$) (fig. 2), decrease of oxygen consumption rate ($p<0.01$) (Fig. 3), increase of glucose disposal ($p<0.01$) (Fig. 4), of lactate production ($p<0.001$) (Fig. 5) and of G6PDH and GR activity ($p<0.01$) (Fig. 6). Moreover this monomer induced a decrease of GSH and total GSH concentrations ($p<0.05$) (Fig. 7).

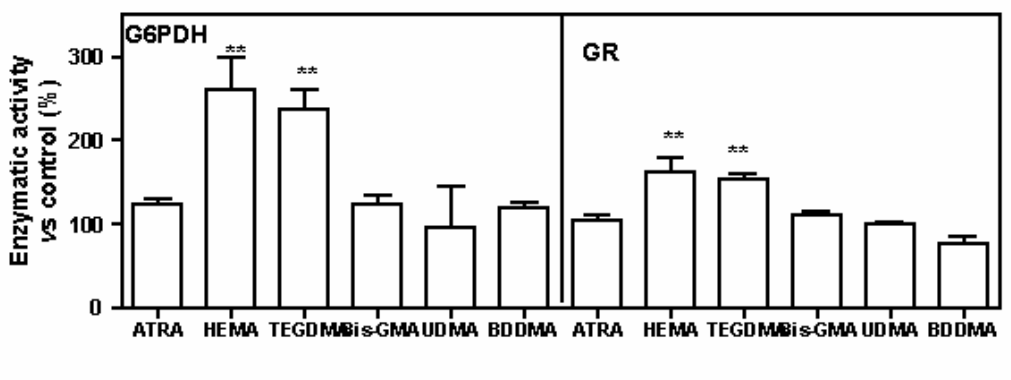

Figure 6. G6PDH and GR activity of HL-60 cells: untreated and treated with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L). The results were reported as percentage of enzymatic activity of treated cells vs control; ** ($p<0.01$) significantly different from ATRA. Further details in Materials and methods.

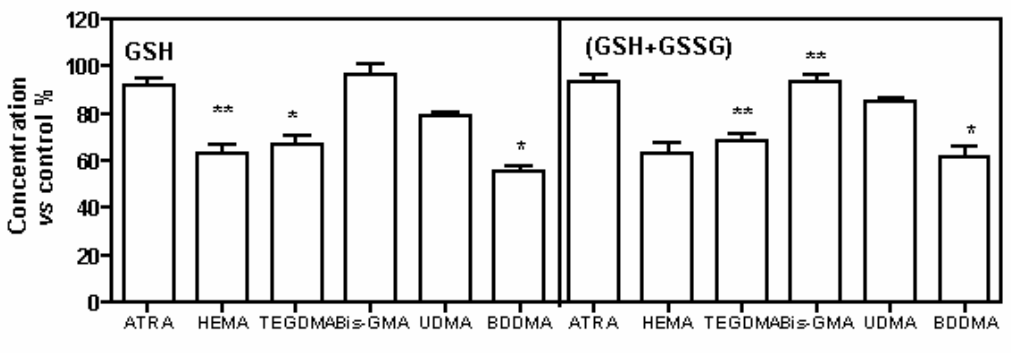

Figure 7. Concentration of reduced glutathione and total glutathione in HL-60 cells, untreated and treated with ATRA (0.001 mmol/L), HEMA (1.1 mmol/L), TEGDMA (0.38 mmol/L), Bis-GMA (0.016 mmol/L), UDMA (0.055 mmol/L) or BDDMA (0.4 mmol/L) Results are expressed in percentage of treated cells vs control; *($p<0.05$) and ** ($p<0.01$) significantly different from ATRA. Further details in Materials and Methods.

3.3. Bis-GMA

This monomer showed, at a concentration of 0.016 mmol/L, a significant cytostatic effect ($p<0.01$) (Fig. 1) and a cellular differentiation action ($p<0.01$) (Fig. 2); it induced a decrease of oxygen consumption rate in HL-60 cells ($p<0.01$) (Fig. 3) and an increase of glucose disposal ($p<0.01$) (Fig. 4) and of lactate production ($p<0.001$) (Fig. 5). In presence of Bis-

GMA, G6PDH and GR activity (Fig. 6) as well as GSH and total glutathione concentrations remain unaffected (Fig. 7).

3.4. UDMA

UDMA provoked a fall in the cellular proliferation rate compared to control at a concentration of 27.5 x 10^{-3} mmol/L ($p<0.01$) in absence of cellular mortality; at the concentration of 55.0 x 10^{-3} mmol/L UDMA still caused a strong decrease of the cellular proliferation rate ($p<0.001$), although strongly increasing cellular mortality ($p<0.01$) (Fig. 1); at the latter concentration the monomer induced in HL-60 cells a recovery of oxidative burst (Fig. 2) and a significant reduction of oxygen consumption rate ($p<0.05$) (Fig. 3).

HL-60 cells treated with this monomer display a statistically significant increase of glucose consumption ($p< 0.01$) (Fig. 4) and of lactate production ($p<0.01$) (Fig. 5), but they did not show any statistically significant alteration of the enzymatic activity ($p>0.05$) (Fig. 6). No change in GSH and in total GSH concentrations is observed, probably because of the very low UDMA concentration employed (Fig. 7).

3.5. BDDMA

BDDMA (0.2 mmol/L) significantly decreased the cellular proliferation rate ($p<0.01$) in absence of cellular mortality, while at 0.4 mmol/L concentration it induced a significant decrease of cellular proliferation rate ($p<0.01$) with an increase of cellular mortality ($p<0.01$) (Fig.1). Chemiluminescence analysis showed that this monomer (0.4 mmol/L) produces a recovery of oxidative burst in HL-60 cells (Fig. 2). BDDMA caused a significant reduction of oxygen consumption rate ($p<0.05$) (Fig. 3) and a significant increase of glucose consumption ($p< 0.01$) (fig.4) and of lactate production ($p<0.01$) (Fig. 5). Monomer treated HL-60 cells did not show any statistically significant alteration of the enzymatic activity ($p>0.05$) (Fig. 6) while a significant decrease of both GSH and GSH + GSSG levels ($p<0.01$) is observed (fig.7).

3.6. ATRA and DMSO

ATRA displayed, at the concentration used, the well known cytostatic and differentiating effects (Figs.1 and 2) while the other parameters evaluated in this study resulted completely unaffected. DMSO (0.1%) did not induce any effect on the cells (data not shown) as already reported [33].

4. DISCUSSION

The composite resins—introduced in restorative dentistry to compensate for the aesthetical and biocompatibility problems presented by the amalgam fillings—have been

worldwide utilized during the last decades for adult and young patients: a careful evaluation of the interactions between the components of these materials and the host becomes therefore important.

Over the years, the compatibility concept evolved from simple acceptability by the surrounding tissues and by the whole organism to appropriate host response, taking therefore into account any cytotoxic effect and its biochemical causes [34].

In vitro tests are especially suitable for this purpose allowing the separate analysis of the different metabolic aspects, whereas the same results could not be obtained through in vivo trials. As soon as it was realized that the polymerization of methacrylates also present in dental composite resins did never reach completion, it became evident the necessity to appraise the biocompatibility of the monomers leached from composite resins.

Our data indicate that all the methacrylic monomers affect the metabolism of HL-60 cells, each at different concentrations and in different ways, at the same time eliciting cellular differentiation. Experiments on metabolism of cells treated with ATRA—a well-known differentiating agent [35]—allowed to establish unerringly whether the metabolic alterations were triggered by the monomers or by the cellular differentiation process. In this regard, the recovery of the respiratory burst—due to a correct assembly of the membrane NADPH oxidase system—was used as a cellular differentiation marker. Although the biological mechanism of differentiation has not been ascertained, two hypotheses can be however assumed as the most reasonable ones: a) ROS produced by damaged mitochondria can act as second messengers in the signal transduction pathway [36] or b) methacrylates could supposedly behave as unspecific differentiating agents [37].

The oxygen consumption rate is a typical parameter for mitochondrial function and its measure allows the evaluation of the metabolic state of the cells, including a widespread oxidative stress: our data suggest that all the utilized monomers elicit a slowdown of cellular respiration. Considering the linkage between glucose metabolism and mitochondrial function, the increase of glucose disposal in HL-60 cells treated with TEGDMA, Bis-GMA, UDMA or BDDMA monomers is present as expected, whilst it is intringly lacking in HEMA-treated cells.

Moreover, lactate concentration (a parameter for anaerobic glycolysis) and G6PDH enzymatic activity (the HMP shunt rate-limiting step) have been assessed to ascertain the metabolic pathway through which glucose is disposed. Indeed, the glycolytic pathway increases steeply as a response to the energy shortage resulting from mitochondrial damage. On the other hand, HMP shunt is stimulated to produce more NADPH, which is required for the reduction of GSSG back to GSH. At this regard, TEGDMA enhances both the metabolic pathways, whereas Bis-GMA, UDMA and BDDMA increase only the anaerobic glycolysis and HEMA only the hexose monophosphate shunt, respectively. Consequently, TEGDMA, UDMA, BDDMA and Bis-GMA monomers elicit an increase in glucose consumption mainly by stimulating the glycolitic pathway, driving the cells to meet the energy demand *via* glycolysis. TEGDMA and HEMA monomers trigger the HMP shunt to meet the increased oxidative stress determined by the incomplete reduction of oxygen and consequent enhancement of ROS generation in damaged or less efficient mitochondria [38-41]. The NADPH increase in TEGDMA- and HEMA-treated cells is most probably due to the increased activity of NADPH-dependent Glutathione Reductase, an adaptive response to counterbalance GSH oxidation.

As previously stated, both the direct interaction of GSH with HEMA, BDDMA and TEGDMA and/or the oxidative stress induced by them can reduce the cellular GSH concentration. Therefore, as it is suggested also by a decrease in the GSH + GSSG amount, such a phenomenon could originate mainly from a direct reaction between methacrylic monomers and GSH [26] and, to a lesser extent, from an oxidation induced by ROS, as indicated by an increased the GR activity (not observed in presence of BDDMA). On the other hand, Bis-GMA and UDMA do not alter GSH concentration, in line with the lack of modification of the related enzymatic activities probably because of the very low concentrations we were compelled to use here for their very hight cytotoxicity.

CONCLUSION

The alterations in energy metabolism and glutathione balance—produced in the cellular metabolism by the monomers under study—could be considered one of the mechanisms inducing clinical and sub-clinical adverse effects sometimes reported during the use of dental resins.

Our opinion is that such investigations can therefore be helpful to test the behaviour of materials designed to be applied inside the human body.

REFERENCES

[1] Geurtsen W. Substances released from dental resin composites and glass ionomer cements *European Journal of Oral Science,* 1998; 106: 687-695.

[2] Geurtsen W, Spahl W, Leyhausen G. Variability of cytotoxicity and leaching of substsances from four light-curing pit and fissure sealants. *Journal of Biomedical Materials Research,* 1999; 44: 73-77.

[3] Ferracane JL Current trends in dental composites. *Critical Reviews in Oral Biology and Medicine*,1995; 6: 302-318.

[4] Stein PS, Sullivan J, Haubenreich JE, Osborne PB. Composite resin in medicine and dentistry. *Journal of long-term effects of medical implants*, 2005;15(6):641-654.

[5] Bouillaguet S, Wataha JC, Hanks CT, Ciucchi B, Holz J. In vitro cytotoxicity and dentin permeability of HEMA. *Journal of Endodontics,* 1996; 22: 244-248.

[6] Bouillaguet S, Virgillito M, Wataha J, Ciucchi B, Holz J. The influence of dentine permeability on cytotoxicity of four dentine bonding systems, in vitro. *Journal of Oral Rehabilitation,* 1998; 25: 45-51.

[7] Gerzina TM, Hume WR. Diffusion of monomers from bonding resin-resin composite combinations through dentine in vitro. *Journal of Dentistry,* 1996; 24:125-128.

[8] Jaffer F, FinerY, Santerre JP. Interactions between resin monomers and commercial composite resinswith human saliva derived esterases. *Biomaterials*, 2002; 23: 1707-1719

[9] Geurtsen W, Lehmann F, Spahl W, Leyhausen G. Cytotoxicity of 35 dental resin composite monomers/additives in permanent 3T3 and three human primary fibroblast cultures. *Journal of Biomedical Materials Research*, 1998; 41:474-480.

[10] Geurtsen W. Biocompatibility of resin-modified filling materials. *Critical Review in Oral Biology and Medicine,* 2000; 11(3): 333-355.

[11] Goldberg M. In vitro and in vivo studies on the toxicity of dental resin components: a review. *Clinical Oral Investigations*, 2008;12(1):1-8.

[12] Hensten-Petterssen A. Skin and mucosal reactions associated with dental materials. *European J*ournal of *Oral Sciences,* 1998; 106:707-712

[13] Schuster GS, Erbland JF, Lefebvre CA, Caughman GB and Knoernschild KL. Effects of an aminomethacrylate on epithelial cell lipid metabolism. *Journal of biomaterials science. Polymer edition*, 1997; 8 (5): 363-375.

[14] Engelmann J, Leyhausen G., Leibfritz and Geurtsen W. Metabolic effects of dental resin components in vitro detected by NMR spectroscopy. *Journal of Dental Research*, 2001; 80(3):869-875.

[15] Geurtsen W, Leyhausen G. Chemical-biological interactions of the resin monomer triethyleneglycol-dimethacrylate (TEGDMA). *Journal of Dental Research,* 2001; 80 (12):2046-2050.

[16] Lefeuvre M, Amjaad W, Goldberg M, Stanislawski L. TEGDMA induces mitochondrial damage and oxidative stress in human gingival fibroblasts. *Biomaterials* 2005; 26(25):5130-5137.

[17] Engelman J, Janke v, Volk J, Leyhausen G, Von Neuhoff N, Schlegelberger, Geurtsen W. effects of Bis-GMA on glutathione metabolism and apoptosis in human gingival fibroblasts in vitro. *Biomaterials*, 2004; 25: 4573-4580.

[18] Chang HH, Guo MK, Kasten FH, Chang MC, Huang GF, Wang YL, Wang RS, Jeng JH. Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. *Biomaterials* 2005; 26(7):745-753.

[19] Nocca G, De Palma F, Minucci A., De Sole P,. Martorana GE, Callà C, Morlacchi C, Gozzo ML,. Gambarini G, Chimenti C, Giardina B and Lupi A.. Alterations of energy metabolism and glutathione levels of HL-60 cells induced by methacrylates present in composite resins *Journal of Dentistry* 2007; 35 (3):187-194.

[20] Noda M., Wataha JC, Kaga M, Lockwood PE, Volkmann KR, Sano H. Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentration. *Journal of Dental Research,* 2002; 81: 265-269.

[21] Lefeufre M, Bourd K, Loriot M-A, Goldberg M, Beaune P, Périanin A. TEGDMA modulates glutathione-S-transferase P1 activity in gingival fibroblasts. 2004; *Journal of Dental Research*, 83:914-919.

[22] Kaga M, Noda M, Ferracane JL, Nakamura W, Oguchi H, Sano H. the in vitro cytotoxicity of eluates from dentin bonding resins and their effect on tyrosine phosphorylation of L929 cells. *Dental Material*, 2001; 17:333-339.

[23] Brand KA, Hermifisse U. Aerobic glycolisis by proliferating cells: a protectivee strategy against reactive oxygen species. *FASEB Journal* 1997; 11(5):388-395

[24] Kletzien RF, Harris PK, Foellmi LA. Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB Journal* 1994; 8: 174-181.

[25] Zimmer HG Regulation of and intervention into the oxidative pentose phosphate pathway and adenine nucleotide metabolism in the heart. *Molecular and Cellular Biochemistry* 1996; 160-161: 101-109.

[26] Geurtsen W, Leyhausen G. Chemical-Biological interactions of the resin monomer triethyleneglycol-dimethacrylate (TEGDMA). *Journal of Dental Research,* 2000; 80 : 2046-50.

[27] Scatena R, Bottoni P, Vincenzoni F, Messana I, Martorana GE, Nocca G, *et al* Bezafibrate induces a mitochondrial derangement in human cell lines: a PPAR-independent mechanism for a peroxisome proliferator. *Chemical Research in Toxicology,* 2003; 16: 1440-1447.

[28] Terasaka H, Kadoma Y, Sakagami H, Fujisawa S. Cytotoxicity and apoptosis-inducing activity of bisphenol A and hydroquinone in HL-60 cells. *Anticancer Research* 2005; 25: 2241-2248.

[29] Scatena R, Nocca G, De Sole P, Rumi C, Puggioni P, Remiddi F, Bottoni P, Ficarra S, Giardina B. Bezafibrate as differentiating factor of human myeloid leukemia cells. *Cell Death and Differentiation,* 1999; 6(8):781-787.

[30] De Baetselier P, Schram E. Luminescent bioassay based on macrophage cell lines. *Methods in Enzymology*, 1986; 133:507-530.

[31] Ellman G, Lysco H. A precise method for the determination of whole blood and plasma sulfhydryl groups. *Analytical biochemistry,* 1979; 93(1):98-102

[32] Wataha JC, Lewis JB, Lockwood PE, Rakich DR Effect of dental metal ions on glutathione levels in THP-1 human monocytes. *Journal of Oral Rehabilitation,* 2000; 27: 508-516.

[33] Yeh CJ, Papamichael M, Faulk WP. Loss of transferrin receptors following induced differentiation of HL-60 promyelocytic leukemia cells. *Experimental cell research,* 1982; 138(2):429-433.

[34] Schmalz G. Use of cell cultures for toxicity testing of dental materials: adavantages and limitation. *Journal of Dentistry,* 1994; Suppl 2, 22: S6-S11.

[35] Zhang JW, Wang JY, Chen SJ, Chen Z. mechanisms of all-trans retinoic acid-induced differentiation of acute promyelocytic leukemia cells. *Journal of Biosciences,* 2000; 25: 275-284

[36] Forman HJ, Fukuto JM, Torres M. Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *American Journal of Physiology. Cell Physiology,* 2004; 287: C246-C256

[37] Santos NC, Figueira-Coelho J, Martins-Silva J, Saldanha C Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochemical Pharmacology,* 2003; 65, 1035-1041.

[38] Hagen T, D'Amico G, Quintero M, *et al*. Inhibition of mitochondrial respiration by the anticancer agent 2-methoxyestradiol. *Biochemical and Biophysical Research Communications* 2004; 322: 923-929.

[39] Lenaz G. The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. *IUBMB Life,* 2001; 52:159-164.

[40] St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD Topology of superoxide production from different sites in the mitochondrial electron transport chain. *Journal of Biological Chemistry* 2002; 277: 44784-44790.

[41] Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *Journal of Biological Chemistr,* 2003; 278: 36027-36031.

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Chapter 6

COMPARISON OF ANTIBACTERIAL EFFECTS OF DENTIN BONDING AGENTS IN VITRO

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ABSTRACT

This study compared the antibacterial effects of Clearfil Protect Bond (CPB), Clearfil SE Bond(CSB) and Xeno III(XIII) against cariogenic bacteria by disc diffusion method, agar well method and tooth cavity model using demineralized dentin.

For the agar well method, the test materials were filled in the inoculated agar wells. For the disc diffusion method, the paper discs were embedded in adhesives and placed on the agar plates. After 48 h, the inhibition zones were measured in mm. Four cavities were prepared on the dentin surface and demineralized by acid-gel method and left in bacterial suspensions for 3h. The materials were applied on the cavities and the fourth was left unapplied for control. Standart amounts of dentin chips were collected from the cavities and the number of bacterial recovery was counted.

CPB produced the significantly widest inhibition zones against all three bacteria within disc diffusion method and agar well method, where XIII couldn't produce any by disc diffusion method ($p<0.05$). For the tooth cavity model, CPB resulted in significantly less bacterial recovery than the other adhesives used on the demineralized dentin ($p<0.05$).

Within all the methods used, CPB was found to have the most antibacterial effects against the three bacteria.

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INTRODUCTION

Caries development is a dynamic process between demineralization and remineralization resulting in cavitation [1]. While removing this carious lesion, bacteria may remain in dentinal tubules even the caries detectors and clinical parameters of dentin hardness and color are supporting the complete elimination of microorganism in the cavity [2,3].In the past, carious dental tissue was excavated by extensive surgical excision during which all diseased tooth structures had to be removed. The philosopyhy of surgical excision has greatly changed to a concept of minimally invasive techniques by the development of the functional and effective dentin bonding systems [4].

Minimal invasive dentistry is focused on less surgical intervention that is preserving sound tooth structures as much as possible [5,6]. Since the bacterially contaminated, demineralized and caries effected dentin is to be removed, after minimally invasive caries intervention, some pioneer bacteria are found in the reminerazable, caries affected layer [7]. This bacterial presence and the leakage through the interface between the restoration and the cavity walls may be involved in the development of recurrent caries and subsequently result in pulpal inflammation [8,9].

Dental adhesive systems are materials developed to improve the adhesion between restorative composite resins and dentin [10]. Adhesion also reduces microleakage at the restoration-tooth interface. Preventing this ingress of oral fluids and bacteria along the cavity wall reduces most clinical problems such as post operative sensitivity, marginal staining, and recurrent caries, all of which are known to jeopardize the clinical longevity of restorative efforts [11,12].

Recent dentin adhesives are classified according to the interaction with the dentin smear layer; the total-etch technique or the self-etch technique [13]. In total etch dentin bonding systems phosphoric acid is used to etch dentin and enamel before applying the primer or primer and adhesive together in one botttle. While etching dentin, the smear layer is removed and dentinal tubules are opened. Self etch adhesives , which do not have a separate acid-etch step; do not remove the smear layer completely. These adhesives condition and prime enamel and dentin simultaneously without rinsing and dissolve hydroxyapatite partially to yield a resin-infiltrated zone with incorporated minerals [13]. Self-etch adhesives have been classified in three categories: mild, such as Clearfil SE Bond and Clearfil Protect Bond, moderate, and aggresive such as Xeno III [14]. Most of the self-etch primers are composed of acidic functional monomers, generally phosphoric-acid esters, with a pH relatively higher than that of phosphoric-acid etching gels [15].

During the bonding procedures etching with an acidic solution may be effective in reducing the number of the bacteria left in the cavity [16]. Cavity cleansing effect of water rinsing following acid application is limited [17]. Since the self etching systems does not remove the smear layer, residual bacteria can be anticipate in the deeper dentin layer. Adhesive systems that have antibacterial effects can provide treatment to eliminate harmful effects caused by bacterial presence, leakage along the cavity wall and the restoration. The use of the materials with inhibitory effects on microbial growth may also help alleviate postoperative sensitivity and extend the longevity of the restorations [7].

The resin monomer 12-methacryloyloxydodecyl-pyridinium bromide, known as MDPB is one of the antibacterial agents incorporated into adhesive materials [7,18,19]. The main

advantage of MDPB is that it can copolymerize with other resin monomers within the polymer matrix, which confers safety and prolonged antibacterial activity to this agent. MDPB does not leach out from the material and maintains a good survival rate for the restoration, and also, unlike soluble antibacterial agents, it does not adversely effect the physical and mechanical properties of the materials to which it is incorparated [18-20]. Imazato et. al reported that the dentin primer incorporating MDPB has strong bactericidal effect against oral bacteria .The incorporation of MDPB into self etching primer has been adventageous since the primer is directly applied to the dentin to inhibite the microorganisms remaining inside the dentin tubules [21-23].

This study aimed to compare the antibacterial effects of Clearfil Protect Bond, Clearfil SE Bond and Xeno III over the demineralized dentin using agar disc diffusion, paper disc-diffusion test. The hypothesis that the Clearfil Protect Bond primer disinfects cavity was also examined by in vitro tooth cavity model using demineralized tooth cavity surfaces containing bacteria.

MATERIALS AND METHODS

The components and the manufacturers of the materials used in the study are listed in Table 1. The antibacterial effects of the materials were examined using these bacterial strains; *Streptococcus Mutans ATCC 25175*, *Lactobacillus casei ATCC 4646*, and *Actinomyces naeslundii ATCC 19246*. The techniques used in the study are agar well-diffusion method, paper disc- diffusion method and the tooth cavity model.

Agar-Well Method

Antibacterial effects of Clearfil Protect Bond, Clearfil SE Bond, Xeno III against three bacterial species; *Streptococcus mutans* NCTC10449, *Lactobacillus casei* ATCC4646, and *Actinomyces naeslundii* ATCC19246 were compared by the agar well method. % 0.2 Chlorhexidine gluconate solution were as the positive control group.

Each bacterial strain was cultivated in 5 ml of BHI broth (Brain Heart Infusion , BHI^{TM} , Merck, Darmstadt, Germany) for 24 h at 37° C for *S. mutans* and 48 h for *L. casei* and *A. naeslundii.* BHI agar was evenly distributed over the surface of petri dishes to a thickness of 5mm. 0.1 ml of *S. mutans ATCC 25175* suspension was inoculated by a sterile glass rod over the agar surfaces. Five wells with diameters of 4 mm were punched into the agar wells using the end of a sterile Pasteur pipette.

The first well was filled with 20 µl Clearfil Protect Bond primer, the second well was filled with 20µl Clearfil SE Bond primer, for the third well equal amounts of Xeno III bond and primer was mixed and inserted in the well, the forth well was filled with equal amounts of Xeno III primer and bond then light cured with a halogen light curing unit for 10 s (Hilux 350, 230 V-50/ 60 hz,Benliolu,Turkey) and the last well was filled with 20µl chlorhexidine gluconate for the control group. Plates were incubated unaerobically for 48 h at 37° C. The size of inhibition zones for each material was measured by the inhibition halo diameters occured around the wells filled with the test materials;

Size of inhibition zone=(diameter of halo-diameter of well)x1/2

Disc Diffusion Method

The agar surfaces were inoculated by the three bacteria as mentioned above. 20 µl of each test material wae pipetted over the sterile paper discs with a 6 mm diameter and 1,5 mm thickness (54991 Disques non imprégné, bioMerieux SA, Marcy l'etoile, France) . Discs impregnated with Xeno III were light cured for 10 seconds and the other Xeno III group was applied without light cure. Then the discs were placed on the inoculated agar surfaces by sterile tweezers. The plates were incubated for 24 h at 37° C and the inhibition zones produced around the paper discs were calculated in milimeters.

The agar diffusion and the disc diffusion tests were applied according to the method described above for the other bacterial strains; *L. casei* ATCC 4646, and *A. naeslundii* ATCC 19246.

The agar diffusion and the disc diffusion tests were repeated 10 times for all bacterial strains.

Tooth Cavity Model

Thirty-six freshly extracted human non-carious third molars were used for the tooth cavity model. The teeth were cleaned away from debris with a plastic periodontal curette and tap water and then stored in sterile physiological saline (SPS) until use within one month from extraction. They were randomly divided into three groups of 12 teeth for each bacterial strain. The groups tested were: group 1, *S. mutans* ATCC ; group 2, *L casei*; group 3, *A. naeslundii*. The occlusal enamel part (±3 mm) of the crown was removed with water cooled, slow-speed (4000 r.p.m.) diamond bur was used to obtain flat dentinal surfaces (Isomet 1000; Buehler, Lake Buff, IL, USA). Four cylindrical cavities were prepared (diameter 1 mm, depth 2 mm) on the occlusal surface of each tooth without causing pulp exposure. Additionally, the apicies of the teeth were sealed with glass ionomer cement. The teeth surfaces apart from a 1mm away from cavity cavomargins were coated 4 times with nail polish. Each tooth was taken to the 100 ml of sodium carboxymethylcellulose gel (CMC; Fluka, CMC Na salt;Medium Viscocity, Sigma –Aldrich Chemie, Sweden) made up with lactic acid buffer at pH 4.0 for 4 weeks with the solution being changed every 2 weeks at 37° C to produce demineralized cavity surfaces. After demineralizing procedure, the teeth were washed with sterile water and taken into sterile tubes containing SPS to wash out the gel medium for 24 hours at 37° C. The teeth then sterilized by an autoclave for 15 min at 121° C and later on they were taken into BHI broth and incubated for 24 h at 37° C to confirm the sterility. Following confirmation the teeth were washed out the culture medium by taking into individual tubes filled with 2 ml of SPS and kept for 24 h at 37° C. After drying with sterile paper points the teeth were placed in sterile tubes containing 3 ml of S.mutans suspension (approx. $1x10^6$) and left for 3 hours to obtain bacterial colonization into demineralized dentin to simulate carious dentin surfaces. The teeth were then taken out from the tubes and dried with sterile paper points.

Three of the cavities in each tooth were used as experimental cavities and one as a control for only demineralized infected dentin.

The three cavities of each tooth were treated according to the group to which they belonged: the first cavities were treated with Xeno III, the second and the third cavities were

treated with Clearfil SE Bond and Clearfil Protect Bond respectively, according to the manufacturer's instructions. Equal amounts of Xeno III primer and bond dropped and mixed for 5 seconds, applied for 10 seconds and light cured for 10 seconds; and for Clearfil SE Bond and Clearfil Protect Bond, primer was applied for 20 s, air blowed gently, and then bond was applied and light cured for 10 s. The fourth cavity was left untreated for the control group. After the bonding system applications, each cavity was filled with a piece of a sterile sponge (Kuraray Liner Bond) and a fluoride and eugenol free temporary filling material. The teeth were kept separately in SPS at 37°C for 72 h. The temporary fillings and sponges were removed using sterile excavators and sterile tweezers without coming into contact with the dentin walls of the cavity. Dentin chips were collected with cooled round shaped tunsten carcide burs from the surrounding walls of each cavity and collected into sterile tubes. The dentin chips were weighed and then diluted 1 : 100 in PYB medium. The solution was stirred for 30 s and a series of 10-fold dilutions was prepared. The numbers of *S. mutans*, *L. casei* and *A. naeslundii* (CFU/ml) were determined by viable plate counting on sheep Blood Agar.

Statistical Analysis

The one way ANOVA test was used to evaluate the antibacterial activity of the dentin bonding systems compared by the agar well and the disc diffusion methods at a significance level of $p<0.05$. The tooth cavity model results were subjected to Dunnette C test at a significance level of $p<0.05$.

RESULTS

The inhibition zone sizes of test materials (dentin bonding systems and chlorhexidine) produced against all three bacteria are shown in Table 1.

Agar Well Diffusion Method

Clearfil Protect Bond produced the largest inhibition zone against *S.mutans* ($p<0,05$) and it is followed by Chlorhexidine,Clearfil SE Bond and Xeno III specimens respectively (Table 2). Xeno III with light activation produced the smallest inhibition zones with a significant difference between other test materials ($p<0,05$).

The largest inhibition zone against *L.acidophilus* was shown by Clearfil Protect Bond while Clearfil SE Bond and Chlorhexidine produced smaller inbition zones respectively. Light cured Xeno III produced the smallest inhibition zones with a significant difference ($p<0,05$).

Clearfil Protect Bond produced the greatest antibacterial activity against *A.naeslundii* ($p<0,05$).Clearfil SE Bond and chlorhexidine produced smaller inhibition zones where the light cured Xeno III showed the least antibacterial effect ($p<0,05$).

Disc Diffusion Method

Clearfil Protect Bond showed the greatest antibacterial effects among the test materials while Xeno III specimens with or without light activation did not produce any inhibition zone against *S.mutans* ($p<0,05$) (Table 2).

Chlorhexidine produced larger inhibition zones than Clearfil Protect Bond and Clearfil SE Bond with a significant difference ($p<0,05$) but Xeno III couldn't show any antibacterial effects against *L.casei* in both ways applied.

Clearfil Protect Bond, Clearfil SE Bond and Chlorhexidine showed antibacterial effects against *A.Naeslundii* by producing inhibition zones respectively but the size of the zones produced did not differ significantly ($p>0,05$) while Xeno III couldn't produced any inhition zone.

Tooth Cavity Model

The number of bacterial recovery of the infected tooth cavities are listed at Table 3.

Clearfil Protect Bond showed the greatest antibacterial effect by the least bacterial recovery among the dentin bonding systems compared but all the dentin bonding systems tested in tooth cavity model inhibited *S.mutans, L.casei* and *A.naeslundii* significantly when compared with empty cavities **(**$p<0,05$**).**

DISCUSSION

One-bottle dentin bonding systems which does not need a separate etching and rinsing step are desired to have an efficient antibacterial activity [18]. The antibacterial activity may be effective to inactivate the residual bacteria remaining in the cavity and contribute the remineralization of the caries affected dentin [24].

There are several methods to evaluate the antibacterial properties dental materials. The antibacterial effects of dentin bonding systems are generally measured by using agar diffusion methods. The agar diffusion methods are widely used since they are simple to handle and economic to determine the antibacterial effects of liquid substances. Results of the agar diffusion tests; inhibition zones indicate the amounts of the antibacterial materials that diffuse through the hidrophylic agar .The diffusion rate and ability of the material through the agar medium, contact of the material and the agar surface, bacterial concentration of the agar medium, temperature of the medium are the factors that can be the disadvantages of agar diffusion methods [25]. The inhibition zones produced around the materials indicate the bacteriostatic actions of the samples but not bactericidal actions. The bactericidal effects of the materials can not be indicated by the agar diffusion methods [26].

It is difficult to compare the antibacterial activity of different dentin bonding systems with only agar diffusion methods. Since the diffusion rate of the antibacterial material through the agar varies, diffusion of the antibacterial compound of dentin bonding system applied to dentin also into dentin may differ. The solubility and the diffusion of the antibacterial components such as gluteraldehyde, chlorhexidine and acidic molecules in to dentin are

limited so that the bacteriostatic or bactericidal effects can not be determined [27]. Artificial caries models containing bacterial demineralization can simulate the clinical oral situation. We applied the tooth cavity model using the demineralized teeth to evaluate the clinical-like antibacterial effects of the dentin bonding system which can penetrate through the deeper demineralized dentin layers.

An in vitro tooth cavity model using bovine teeth was developed by Ohmori et al. in order to evaluate the antibacterial activity of dentin primers relevant in the clinical use [26]. To obtain sensitive results, tooth cavity model in which the amounts of dentin chips collected from the infected dentin cavities can be standardized and the type of the bacteria can be chosen by the investigator should be used with agar diffusion methods to simulate the clinical application.

In the present study it was shown that Clearfil Protect Primer produced significantly the largest inhibition zones against *S.mutans*, *L casei* and *A. naeslundii* in the agar well and agar-disc diffusion test. Also, the inhibition zone around of CSEB bond was markedly wider compared with that of Xeno III with and without light curing in agar well test. On the other hand, Xeno III with and without light curing did not produce inhibition zones in disc diffusion test. Adhesion-promoting acidic monomers are elements which support antibacterial effects of dentin primers. Addition of these monomers in large amounts as self-etching primers can decrease pH values of materials enough to kill or at least inactive the bacteria [28]. Phenyl-P, 4-META and MDP are the main acidic monomers contained in the formulation of self-etcing adhesive systems and are capable of demineralizing and infiltrating simultaneously the dentin substrate. According to Ohmori et al. MDP has a higher inhibitory action against microorganisms than Phenyl-P [26]. In the present study, MDP is present both in the primer and in the adhesive components of CPB nd CSEB. However, the inhibition zone of CPB against the tested bacterial strains was markedly wider compared with that of CSEB. In addition to MDP, primer and in the adhesive components of CPB contain another resin monomer, known as MDPB, which is an antibacterial agent composed of a quaternary ammonium with a methacryloyl group.[19] The present results support the findings of other studies which showed that incorporation of MDPB has an additional bactericidal effect compared with other self-etch dentin bonding systems [7,21,29,30]. The incorporation of MDPB into adhesive systems does not interfere with their acidic potential and viscosity [31]. Moreover, the mechanism of the antibacterial effect of quaternary ammonium compound is believed to be due to cationic binding to cell wall components which disturbs the membrane function and subsequently induces leakage of cytoplasmic material [32]. Accordingly, quaternary ammonium causes lysis of bacterial cells and shows strong bactericidal effect. MDPB is a derivative of quaternary ammonium and considered to exhibit bactericidal effect by the similar mechanism as above in unpolymerized form [33]. Dentin primer is applied to the cavity for promoting bonding of restoration to tooth substrate, and it has been suggested that MDPB-containing primer has a potential benefit to prevent harmful effects caused by residual and invading bacteria in the cavity. At the stage before curing, MDPB-containing primer acts as a bactericidal solution and the cavity prepared for restoration is disinfected by unpolymerised MDPB in the primer. On the other hand, in accordance with the findings of previous studies [29,30], in spite of containing fluoride and having the lowest pH (pH 1) among the materials evaluated in the present study, Xeno-III has a greater viscosity due to the presence of UDMA in its formulation, which is a hydrophobic resin monomer with high

molecular weight. Therefore, it is speculated that the viscosity may modulate the antibacterial activity of this material [30].

In the present study, Clearfil Protect bond was found to be the most antibacterial material when applied on the demineralized dentin with the least bacterial recovery against the three bacteria tested. Clearfil SE Bond also showed some antibacterial activity with killing more bacteria then Xeno III. The sequence of the number of the bacteria killed by the dentin bonding systems did not differ when applied to dentin infected by *S.mutans*, *L.casei* and *A.naeslundii* separately. When the number of *L.casei* recovery was evaluated, more bacteria were survived especially by Clearfil SE Bond and Xeno III when compared to the other bacterial groups. This numerical difference can be explained as the acid resistant characteristics of *L.casei* [31]. Harper and Loesche reported that the pH values which cause 100% killing over a 3-h period was 2.3 for *L.casei* and 3.0 for *S.Mutans* [34].

In the present study, Clearfil Protect Bond was confirmed to be effective for eradicating bacteria in dentin using bacteria-impregnated demineralized dentin. In accordance with a previous study, the present findings support that PB, the antibacterial effect of which are not dependant upon acidity as Clearfil SE Bond and Xeno III but the incorporated MDPB, is advantageous for eliminating bacteria in the infected dentin [7]. Because, the acidity of self-etching primers are buffered by interaction with dentin, and dilution of the solution by dentinal fluid reduces the antibacterial effects [35,36]. Ozer et al compared the antibacterial activity of ABF Primer with MDPB and Reactmer Bond releasing flouride and found ABF primer as the most antibacterial against *S.mutans* when used in the tooth cavity model [37]. Turkun et al applied Clearfil Protect Bond and Xeno III on the dentin surfaces infected by *S.mutans* and evaluated Clearfil Protect Bond with killing more bacteria than Xeno III [30] . Also, the antibacterial activity of Clearfil Protect Bond was compared with cavity disinfectants and the results showed that primer of Clearfil Protect Bond exhibited the greatest antibacterial activity against *S.mutans* by the tooth cavity model used [21]. These results are supporting our findings that Clearfil Protect Bond was found to have the most antibacterial activity among the other materials used. It was reported that the potential to arrest the progress of early root caries by application of an antibacterial dentin-bonding system containing MDPB has three components: (1) blocking the porous structure of dentinal lesion to prevent further decay formation by penetration of dentine-bonding systems; (2) inactivation of the bacteria deep within the lesion by penetration of antibacterial dentine primer; and (3) inhibition of plaque formation on the surface by impregnated antibacterial resin [31]. MDPB can copolymerize with other monomers, and resin-based materials containing MDPB are able to show inhibitory effects on bacterial growth or plaque formation on their surface via the immobilized antibacterial agent [19,38]. In addition, it was reported that MDPB-containing primer revealed an antibacterial effect after being cured, so that the primer impregnated and cured in dentinal lesions should inhibit invading bacteria within the lesion [31,39]. However, to achieve efficient inhibition of plaque formation on the surface of the resin impregnated dentine, it would also be reasonable to incorporate MDPB into the bonding resin [31].

CONCLUSIONS

The results indicated that CPB was the most antibacterial material against *S. mutans, L. casei* and *A. naeslundi* with all the technique used. Furthermore, Clearfil Protect Bond was able to inactivate *S. mutans*, *L. casei* and *A. naeslundii* in the cavity more effectively than Clearfil SE Bond and the fluoride containing adhesive Xeno III. Thus, it can be concluded that incorporation of MDPB into adhesive systems is a potential method for providing antibacterial activity. However, further studies are required to establish its value in clinical situations.

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REFERENCES

Fejerskov, O. Concept of dental caries and their consequences for understanding the disease. *Community Dentistry and Oral Epidemiology* 1997; 25(1):5-12.

Kidd, EA, Joyston-Bechal, S, Beighton, D. Microbiological validation of assessments of caries activity during cavity preparation. *Caries Research* 1993; 27(5):402-8.

Kidd, EA, Joyston-Bechal, S, Beighton, D. The use of a caries detector dye during cavity preparation: a microbiological assessment. *British Dental Journal* 1993; 174(7):245-8.

Sattabanasuk, V, Shimada, Y, Tagami, J. Bonding of resin to artificially carious dentin. *Journal of Adhesive Dentistry* 2005; 7(3):183-92.

Anusavice, KJ. Buonocure Memorial Lecture. Materials of the future: Preservative or restorative. *Operative Dentistry* 1998; 23(4):162-7.

Tyas, MJ, Anusavice, KJ, Frencken, JE, Mount, GJ. Minimal intervention dentistry. A review FDI commision Project 1-97. *International Dental Journal* 2000; 50(1):1-12.

Imazato, S, Kuramoto, A, Takahashi, Y, Ebisu, S, Peters, MC. In vitro antibacterial effects of the dentin primer of Clearfil Protect Bond *Dental Materials* 2006; 22(6):527-532.

Bergenholtz, G, Cox, CF, Loesche, WJ, Syed, SA. Bacterial leakage around dental restorations: its effect on the dental pulp. *Journal of Oral Pathology* 1982; 11(6): 439-50.

Qvist, V. Resin restorations: leakages, bacteria, pulp. *Endodontics & Dental Traumatology* 1993; 9(4):127-52.

Swift, Jr. EJ. Bonding systems for restorative materials. A comprehensive review. *Pediatric Dentistry* 1998; 20(2):80-4.

Phillips, RW. Skinner's Science of Dental Materials. 8th ed. Saunders,Philadelphia, PA; 1982.p.25.

Duke, ES. Adhesion and its application with restorative materials. *Dental Clinics of North America* 1993; 37(3): 329-40.

Perdigao, J. Dentin bonding as function of dentin structure. *Dental Clinics of North America* 2002; 46(2):277-301.

Pashley, DH, Tay, FR. Aggressiveness of contemporary self-etching adhesives. Part II: Etching effects on unground enamel. *Dental Materials* 2001; 17(4):430-44.

Tay, FR, Sano, H, Carvallo, R, Pashley, DH. An ultrastructural study of the influence of acidity of self-etching primers and smear layer thickness on bonding to intact dentin. *Journal of Adhesive Dentistry* 2000; 2(2):83-98.

Settembrini, L, Boylan, R, Strassler, H, Scherer, W. A comparison of antimicrobial activity of etchants used fora total-etch technique. *Operative Dentistry* 1997; 22(2): 84-8.

Luglie, PF, Delitala, PP, Zanetti, S, Sanna, S. An in-vivo bacteriological study on the effects of acid etching at the bttom of the cavities. *Minerva Stomatology* 1998; 47(1-2):19-26.

Imazato, S, Kinomoto, Y, Tarumi, H, Torii, M, Russell, RRB, McCabe, JF. Incorporation of antibacterial monomer MDPB into dentin primer. *Journal of Dental Research* 1997; 76(3):768-72.

Imazato, S, Torii, M, Tsuchitan, Y, McCabe, JF, Russell, RRB. Incorporation of bacterial inhibitor into resin composite. *Journal of Dental Research* 1994; 73(8):1437-43.

Imazato, S, McCabe, JF. Influence of incorporation of antibacterial monomer on curing behavior of a dental resin composite. *Journal of Dental Research* 1994; 73(10):1641-5.

Turkun, M, Turkun, LS, Ergucu, Z, Ates, M. Is an antibacterial adhesive system more effective than cavity disinfectants? *American Journal of Dentistry* 2006; 19(3):166-70.

Jensen, OE, Handelman, SL. Effect of an autopolymerizing sealant on viability of microflora in occlusal dental caries. *Scandinavian Journal of Dental Research* 1980; 88(5):382-8.

Kramer, PF, Zelante, F, Simionato, MR. The immediate and long-term effects of invasive and noninvasive pit and fissure sealing techniques on the microflora in occlusal fissures of human teeth *Pediatric Dentistry* 1993;15(2):108-12.

Lobo, MM, Gonçalves, RB, Pimenta, LAF, Bedran-Russo, AKB, Pereiera, PNR. In vitro evaluation of caries inhibiton promoted by self-etching adhesive systems containing antibacterial agents. *Journal of Biomedical Material Research* 2005; 75(1):122-7.

Tobias, RS. Antibacterial properties of dental restorative materials:a review. *International Endodontic Journal* 1988; 21(2):155-60.

Ohmori, K, Maeda, N, Kohno, A. Evaluation of antibacterial activity of three dentin primers using an in vitro tooth model *Operative Dentistry* 1999; 24(5):279-85.

Palenik, CJ, Setcos, JC. Antimicrobial abilities of various dentin bonding agents and restorative materials *Journal of Dentistry* 1996; 24(4):289-95.

Imazato, S, Imai, T, Ebisu, S. Antibacterial activity of proprietary self-etching primers *American Journal of Dentistry* 1998; 11(3):106-8.

Gondim, JO, Duque, C, Hebling, J, Giro, EMA. Influence of human dentine on the antibacterial activity of self-etching adhesive systems against cariogenic bacteria *Journal of Dentistry* 2008; 36(4):241-8.

Turkun, LS, Ates, M, Turkun, M, Uzer, E. Antibacterial activity of two adhesive systems using various microbiological methods *Journal of Adhesive Dentistry* 2005; 7(4);315-20.

Imazato, S, Walls, AW, Kuramoto, A, Ebisu, S. Penetration of an antibacterial dentin bonding system into demineralized human root dentine in vitro. *European Journal of Oral Sciences* 2002; 110(2):168-74.

Scheie, AA. Modes of action of currently known chemical anti-plaque agents other than chlorhexidine. *Journal of Dental Research* 1989; 68:1609-16.

Imazato, S, Ebi, N, Tarumi, H, Russell, RRB, Kaneko, T, Ebisu, S. Bactericidal activity and cytotoxicity of antibacterial monomer MDPB. *Biomaterials* 1999; 20(9): 899-903.

Harper, DS, Loesche, WJ. Growth and acid tolerance of human dental plaque bacteria. *Archieves of Oral Biology* 1984; 29(10): 843-8.

Itou, K, Torii, Y, Suzuki, K, Nakai, H, Inoue K. Adhesion of restorative resin to tooth-adhesion promoted by Liner Bond II . *Journal of Adhesive Dentistry* 1994; 12:174-81.

Imazato, S. Antibacterial properties of resin composites and dentin bonding systems. *Dental Materials* 2003; 19(6): 449-57.

Ozer, F, Karakaya, S, Unlu, N, Erganis, O, Kav, K, Imazato, S. Comparison of antibacterial activity of two dentin bonding systems using agar well technique and tooth cavity model *Journal of Dentistry* 2003; 31(2):111-6.

Imazato, S, Imai, T, Russell, RRB, Torii, M, Ebisu, S. Antibacterial activity of cured dental resin incorporating the antibacterial monomer MDPB and an adhesion –promoting monomer. *Journal of Biomedical Material Research* 1998; 39(4):511-5.

Imazato, S, Ehara, E, Torii, M, Ebisu, S. Antibacterial activity of dentine primer containing MDPB after curing. *Journal of Dentistry* 1998; 26(3):267-71.

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Chapter 7

PROGRESS TOWARDS BONE AND TISSUE REGENERATION USING PRGF TECHNOLOGY

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ABSTRACT

PRGF technology constitutes a breakthrough in the stimulation and acceleration of soft tissue healing and bone because it allows the local and continuous delivery of a wide range of growth factors and proteins, mimicking the needs of the physiological wound healing and reparative tissue processes. The versatility of this approach facilitates the combination of the different PRGF formulations with autologous bone and even with a wide range of biomaterials. Progress in the development of PRGF technology has stimulated its therapeutic use in numerous medical and scientific fields including dentistry, oral implantology, orthopedics, ulcer treatment, sports medicine and tissue engineering among others.

INTRODUCTION

In the moments after a tissue injury occurs, a large number of intercellular and intracellular pathways are activated and coordinated with the aim of restoring tissue integrity and homeostasis. Cellular and humoral components, the inflammatory pathways and the blood coagulation cascade are activated in addition. A wide range of cells from the injured tissue and adjacent locations undergo marked changes in gene expression and phenotype, leading to cell proliferation, differentiation and migration [1,2]. The development of a new microvasculature and microcirculation is also critical for the homeostasis and correct tissue repair and regeneration. In fact, the absence of a suitable vasculature transporting oxygen,

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nutrients, soluble growth factors and biologically active proteins and numerous cell types to the injured tissue would imply the degeneration of the latter [3,4].

One pivotal discovery that has fuel the research in regenerative medicine and tissue engineering has been the role that cytokines and growth factors play in the process of tissue repair [5]. These molecules provide signals at local injury sites, regulating the mechanisms and pathways that govern wound healing and tissue regeneration [6]. Determining the roles that growth factors play in tissue repair and regeneration is as important as designing, developing and applying suitable formulations that release them with a spatio-temporal control. The latter is especially important as providing the injured tissue a milieu of biological signals may be desirable for the functional and accelerated repair of the tissue [7].

The present chapter describes the significant progress that has been accomplished in the field of platelet-based preparations and especially PRGF technology as a novel approach for growth factor delivery in tissue repair and regeneration. Some of the most interesting therapeutic applications and future challenges and directions in the field are discussed.

NOVEL BIOMATERIALS AND TECHNOLOGIES FOR GROWTH FACTORS RELEASE IN TISSUE REPAIR

Several new biomaterials and biomedical technologies are being explored with the aim of providing a control over growth factor release kinetics. One important challenge in the field has been to produce three dimensional matrices and rendering them deliverable locally through minimally invasive techniques [8,9]. Some of these approaches are based on the combination of the growth factors and autologous, natural or synthetic biomaterials. The combination of polymers and growth factors can provide a controlled release into the local microenvironment to yield desirable concentrations over a period ranging from days to months. The new generation of biomaterials and technologies promises to allow greater control over cell fate and ultimately tissue structure and function. Some examples of polymers used for bioactive factor release include synthetic materials such as poly(glycolic acid) (PLA), poly(lactic acid) (PLG), their copolymers (PLGA) [10] and nitrocinnamate-derived polyethylene glycol (PEG-NC) hydrogel systems [11], natural polymers such as alginate [12] or gelatin [13] and autologous materials such as fibrin [14].

A powerful technology that is gaining the interest of scientists and dentists is plasma rich in growth factor (PRGF) technology. The latter is an evolution of the pioneering studies reported by Marx and Anitua [15,16], in which a preparation rich in platelets and therefore in growth factors was used as a new therapeutic tool to promote bone and soft tissue regeneration. The PRGF technology follows the philosophy and therapeutic objectives of classical platelet-rich products but represents a new exciting alternative to formulate and use platelets in 100% autologous and biocompatible formulations that may circumvent some limitations reported for other platelet rich plasmas.

The step-wise analysis of the main drawbacks reported in the field and the ability to provide a multidisciplinary and integrating overview towards the development of an optimized technology has provided to PRGF technology some important and distinguishing properties when compared to other platelet rich plasmas. Initially, a detailed and careful platelet evaluation and characterization has been realized in order to determine which were

some of the growth factors and proteins contained within the platelets but specially to design easy and simple protocols that facilitate the manipulation and concentration of the cells safely [17,18]. One important advantage of PRGF technology is its versatility as almost 4 different formulations with therapeutic potential from patient's blood by simply controlling the elaboration protocol and coagulation degree of the samples [19,7] (Figure 1).

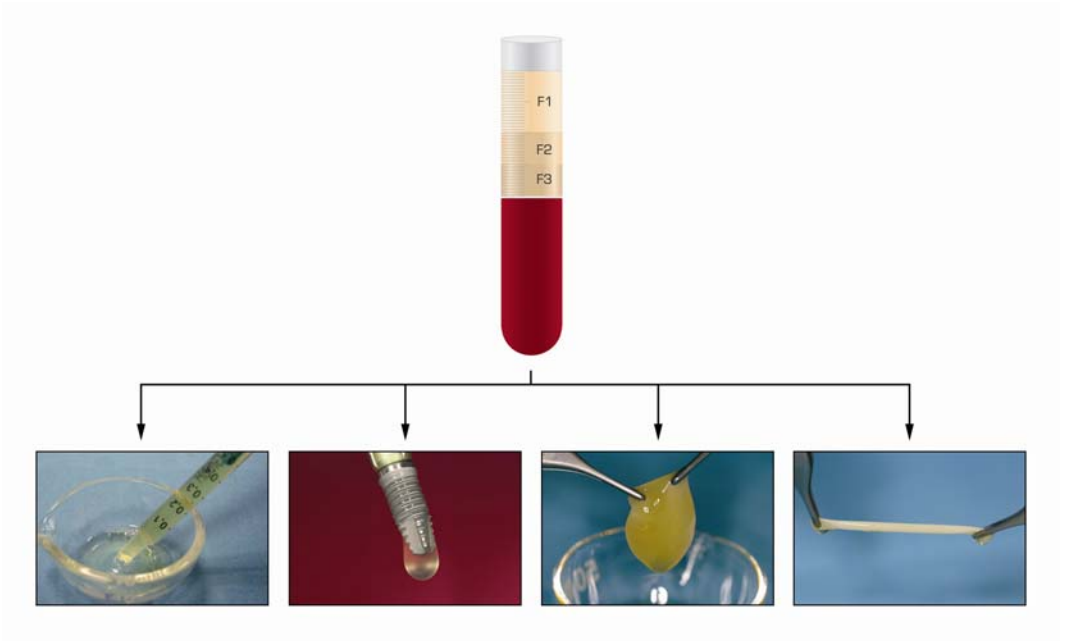

Figure 1. The technology of PRGF enables the production of different formulations with therapeutic potential from the same patient's blood depending on the coagulation and activation degree of the samples. These include the PRGF supernatant, the liquid PRGF for bioactivation of dental implants, the PRGF-scaffold and the elastic fibrin.

The formulations obtained by means of PRGF technology include the PRGF supernatant composed mainly of plasma and platelet growth factors and proteins and used as conventional eye-drop and cell culture media [20], the activated liquid PRGF used for example in surgery, in tissue infiltrations, and to bio-activate dental implants by creating a biologically active nano-membrane on the titanium surfaces [21], the scaffold-like PRGF, a three-dimensional structure composed of fibrillar and cellular components used in ulcer treatment, regeneration of post-extraction defects, and tissue engineering approaches among others and the elastic, dense and haemostatic fibrin which is an excellent tool to seal the post-extraction sockets and to promote the full epithelialization of soft tissues.

Even, a more tightly control over growth factor pharmacokinetics and biodistribution can be obtained if the scaffold-like PRGF is combined with different natural and synthetic biomaterials. For example, by using an acidic gelatin with an isoelectic point of 5.0, the growth factors released by the platelet rich product after its activation are immobilized and retained in the hydrogel through physicochemical interactions. The latter substantially alters growth factor kinetic profile as release will depend on hydrogel degradation [22]. Similar approaches have been described using collagen and calcium sulphate as biomaterials [23,24]. In addition, biomaterials may offer increased stability and strength to the PRGF scaffold

leading to an increased mechanical stability but at the same time the PRGF may facilitate the handling and manipulation of a large number of polymers. For example, in oral implantology, dentists find difficult and challenging the manipulation and application of some bone augmentation materials and even autologous bone. By combining selected biomaterials with scaffold-like PRGF, it is possible to improve the handling and adaptation of the matrix to the injured tissue because the fibrin acts a biologic glue to hold together the matrix particles (Figure 2).

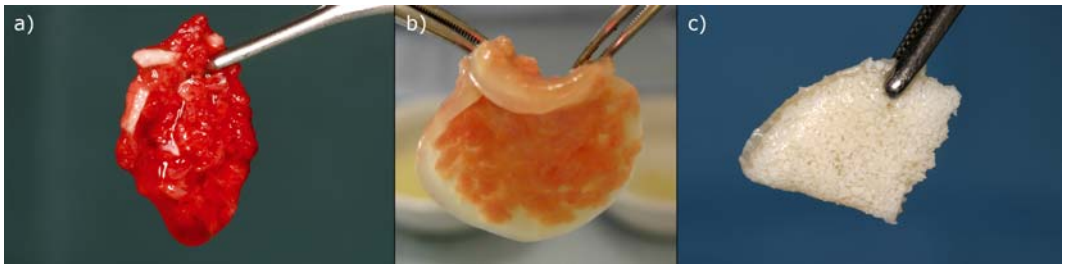

Figure 2. PRGF can be easily combined with a) autologous bone, b) bone allograft particles and c) anorganic bovine bone to improve the handling and manipulation of these biomaterials.

PIVOTAL AND SPECIFIC PROPERTIES OF PRGF TECHNOLOGY

PRGF is prepared following a simple protocol and by means of a single spin method. A reduced blood volume from the patient (10–30 cc) is needed to prepare the different PRGF formulations. The collection and fractionation tubes employed in PRGF elaboration are accepted by the European regulatory agency to be used in the field of tissue engineering, which constitutes a significant step in reinforcing the biosafety and standardization of this technology. Calcium chloride is employed to activate the coagulation cascade and thus the release of growth factors. This is especially interesting as calcium chloride provides a reduced burst effect in comparison with thrombin [25]. In addition, PRGF contains a moderated platelet concentration that has been related with optimal biological benefit [26]. Leukocyte content has been eliminated from PRGF with the aim of avoiding the pro-inflammatory effects of the proteases and acid hydrolases contained in white blood cells and the metalloproteases secreted by leukocytes which may provoke negative destroying effects [27]. Last but not least, the PRGF scaffold creates a three dimensional fibrin structure which maintains the regenerative space and serves as matrix for progenitor cells. This may facilitate the combination of PRGF with mesemchymal stem cells for tissue engineering purposes. Isolated cells, growth factors and biocompatible supporting scaffolds have generally been considered essential prerequisites to tissue engineering approaches. In the last few years, several attempts have been reported especially for bone regeneration, but also for cartilage and periodontal tissue engineering [28-31]. PRGF exerts also potent angiogenic effects [32] and antibacterial effects against *Staphylococcus aureus* and *Escherichia coli* [33].

THE THERAPEUTIC POTENTIAL OF PRGF TECHNOLOGY IN DENTISTRY

The initial evidences of the potential impact of PRGF-expressed growth factors were observed in primary osteoblasts cultured in vitro. Additionally, the growth factors released from PRGF stimulated the proliferation of other cell types including myocites, chondrocites, tenocytes, fibroblasts and even human mesenchymal stem cells. The proliferation induced was significantly higher when compared with the effects exerted by the growth factors alone and even with a combination of them. Others have also observed that growth factors derived from platelets can stimulate the proliferation of different cells including human trabecular bone cells, human osteoblast-like cells, human stromal stem cells and human mesenchymal stem cells [34-38].

Additional scientific evidences show that both the scaffold-like PRGF and liquid PRGF can accelerate and promote bone regeneration and faster osseointegration of dental implants respectively. Briefly, in a first set of experiments artificial defects made in goat's tibiae were carefully filled with PRGF or blood (control). The histological analysis of the biopsies at 8 weeks revealed mature bone trabeculae when PRGF was used to fill the artificial defects and connective tissue with incipient signs of bone formation in the control group [39]. A second study evaluated the potential of PRGF to promote dental implant osseointegration. Twenty six dental implants (13 humidified with liquid PRGF and 13 without PRGF) were placed in the tibiae of goats. Histological and histomorphometrical results demonstrated that bioactivation of dental implants, that is, humidification of implant surface with liquid PRGF increased the percentage of bone-implant contact in 84.7% [21,39]. Moreover, the whole surface of the PRGF-treated implants was covered by newly formed bone whereas only the upper half was surrounded in control implants.

These preliminary evidences suggested potential clinical applications for both the scaffold-like PRGF and the liquid PRGF. In fact, the former may be used for the treatment of post-extraction defects in dentistry, especially when a complete regeneration of the alveolar bone and surrounding soft tissues are totally necessary to ensure the future success of the implant. The liquid PRGF is a valid biological tool to humidify dental implants before their insertion as the bioactivable surface accelerates dental implant osseointegration. These effects can be explained in part by the polarity of the titanium surface and the negatively charged proteins present in the liquid PRGF such as vitronectin and fibronectin. The latter may be adsorbed on the implant's surface, providing specific sites for cell adhesion. Fibronectin, is a well known adhesive protein which will enhance the formation of focal adhesions by osteoblasts [40,41].

The pioneering report that translated the potential of PRGF technology into the clinics was reported in 1999. That initial study involved 20 patients who underwent tooth extraction because of periodontal disease or vertical fractures [16]. The use of PRGF-scaffold improved soft tissue epithelialization and bone regeneration was extensive. The bone tissue of patients receiving PRGF was compact with well-organized trabeculae whereas in the control group only connective tissue and little mature bone were found. Some years later, we have observed when PRGF scaffold was used to fill the post-extraction defects bone regeneration was significantly increased. In fact, filling the defects with PRGF scaffold enhanced bone densitometry within the defect in more than 180%.

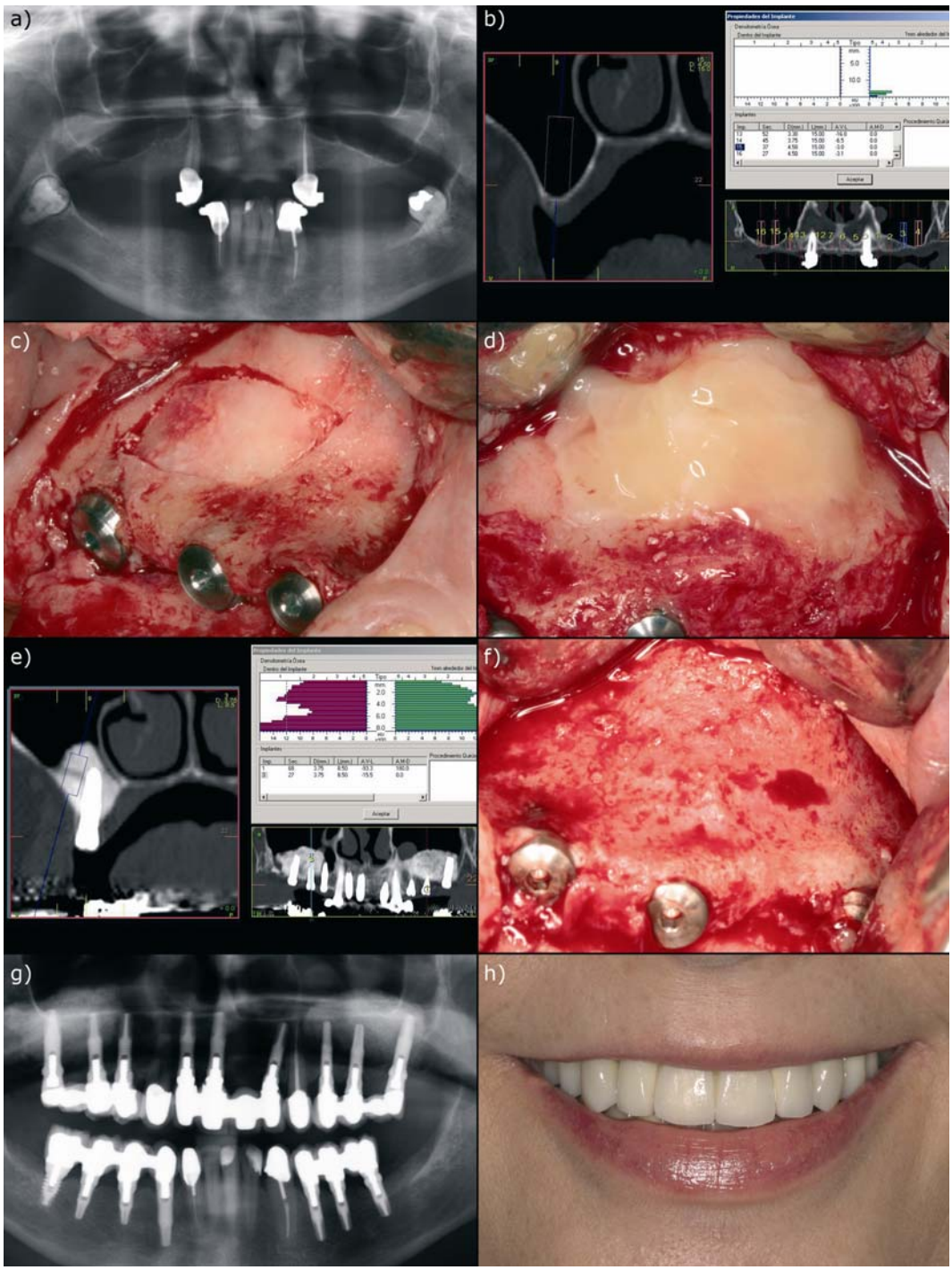

Figure 3. a) Preliminary Rx of a 54 year-old female patient. b) the maxillary CT scan shows us the need for performing bilateral sinus lift. c) The window is closed with the same bone window after filling the space with a mixture of PRGF and anorganic bone. d) the entire defect is covered with a fibrin membrane. e) a CT scan was made six months later to ascertain the condition of the grafts. f) at the reopening six months later, the regenerated bone was clearly observed. g) post-operative radiograph. h) the final aesthetic result.

IMPROVING DENTAL SURGICAL PROTOCOLS AND THE PREDICTABILITY OF DENTAL IMPLANTS

The potential advantages of PRGF technology have been progressively applied to different augmentation protocols such as split and crest expansion [42-44]. Recently, we have reported the benefits that the different PRGF formulation may bring to the sinus elevation protocol. The sinus lateral approach carried out with a periodontal ultrasonic generator enables the separation of the bone window which can be maintained in the pool of biologically active growth factors contained in liquid PRGF. In this way, the viability and functionality of the bone window is maintained until it is placed again in its native anatomic location. In the same approach, the autologous and biocompatible fibrin may be used as autologous sealant biomaterial in the case of Schneiderian membrane perforation and PRGF can be easily combined with any bone augmentation biomaterial to create the final graft. This last combination allows the formation of a clot in which the biomaterial is incorporated and facilitates the manipulation and administration of the latter, increasing the biosafety of the approach (Figure 3).

On the other, recent scientific evidences support the idea that bioactivation of dental surface with liquid PRGF is a useful, safe and predictable approach for dental implant installation. For example, in a study evaluating more than 5700 implants in 1060 patients, the survival of bioactivated implants was higher than 99.2% and only implant staging and the use of special techniques were statistically correlated with lower implant survival rates [45]. In another intriguing 5-year retrospective study, more than 530 short BTI implants humidified with liquid PRGF were evaluated leading to a final survival rate of 99.2% [46]. Last but not least, the retrospective evaluation of more than 1130 immediately loaded bioactivated implants showed a final survival rate of 99.3% with only five implants lost [47].

PRGF TECHNOLOGY IN OTHER MEDICAL AREAS

One important field of research has been the treatment of chronic ulcers. In fact, cutaneous ulceration is actually a common clinical problem rising with the increasing median age of the population. The European Union allocates 2% of the yearly health budget to wound care1 and it is estimated that in the United States the costs related to the care of patients with pressure ulcers is over $1.3 billion per year [48,49]. Absence of healing is not uncommon when predisposing factors such as rheumatism, diabetes, peripheral vascular disease or previous scars are present. In a recent randomized open-label controlled pilot trial, we evaluated the potential of PRGF technology for the treatment of chronic vascular ulcers. By coagulating the PRGF-scaffold in vivo within the bed ulcer and covering afterwards the whole area with a fibrin membrane, we test and compare this autologous approach with the standard therapy [50]. Results showed that at 8 weeks, the mean percentage of surface healed in the PRGF group was 73±22% whereas it was 21±34% in the control group ($P < 0.05$).

Another potential therapeutic target is sports medicine as this field is expanding at a high pace influencing million of people from athletes to people who participate in recreational sport or simply used exercise to stay healthy and active. However, as the number of sport practitioners increases, the rate of sports injuries distressing the musculoskeletal tissues is

also enhancing becoming into a real problem. PRGF has been successfully used in the reconstruction and repair of musculoskeletal tissues. The activated PRGF can be injected among the ruptured tendon fibers after the tendon is sutured. Using this approach, we observed a significant acceleration in functional recovery comparing with a matched group that followed conventional surgery [51]. Encouraging results have already been reported after using PRGF in arthroscopic surgery of anterior cruciate ligament and avulsion of articular cartilage [52].

CONCLUSIONS

PRGF is an optimized approach for the stimulation and acceleration of soft tissue healing and bone regeneration. The specific characteristics and properties of the different PRGF formulations facilitate their application in a wide range of tissues alone or combined with a plenty of biomaterials and even autologous bone. This autologous technology has simplified and improved the overall results of bone augmentation approaches including split, crest expansion, orthognatic surgery and sinus elevation. In the future, novel approaches will be optimized and improved due to the growth factors and biomaterials derived from the PRGF technology.

REFERENCES

[1] Singer, AJ; Clark, RA. Cutaneous wound healing. *N Engl J Med* 1999;341:738-746.

[2] Aarabi, S; Longaker, MT; Gurtner, GC. Hypertrophic scar formation following burns and trauma: new approaches to treatment. *PLoS Med 2*007;4:e234.

[3] Schmid, J; Wallkamm, B; Hammerle, CH; Gogolewski, S; Lang, NP. The significance of angiogenesis in guided bone regeneration. *Clin Oral Implant Res* 1997;8:244-248.

[4] Werner, S; Grose, R; Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* 2003;83:835-870.

[5] Saltzman, WM; Olbricht, WL. Building drug delivery into tissue engineering. *Nat. Rev. Drug Discov.* 2002;1:177-186.

[6] Street, J; Bao, M; deGuzman, L; Bunting, S; Peale, FV Jr; Ferrara, N; Steinmetz, H; Hoeffel, J; Cleland, JL; Daugherty, A; van Bruggen, N; Redmond, HP; Carano, RA; Filvaroff, EH. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. Natl. Acad. Sci.* U.S.A. 2002;99:9656-9661.

[7] Anitua, E; Sánchez, M; Orive, G; Andia, I. Delivering growth factors for therapeutics. Trends. *Pharmacol. Sci*. 2008;29:37-41.

[8] Kikuchi, A; Okano, T. Pulsatile drug release control using hydrogels. *Adv Drug Deliv Rev* 2002;54:53-77.

[9] Kohane, DS; Langer, R. Polymeric biomaterials in tissue engineering. *Pediatr Res* 2008;63:487-491.

[10] Chen, RR; Silva, EA; Yuen, WW; Brock, AA; Fischbach, C; Lin, AS; Guldberg, RE; Mooney, DJ. Integrated approach to designing growth factor delivery systems. *FASEB J.* 2007;21:3896-3903.

[11] Andreopoulos, FM; Persaud, I. Delivery of basic fibroblast growth factor (bFGF) from photoresponsive hydrogel scaffolds. *Biomaterials* 2006;27:2468-2476.

[12] Silva, EA; Mooney, DJ. Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis. *J Thromb Haemost* 2007;5:590-598.

[13] Kikuchi, N; Kitamura, C; Morotomi, T; Inuyama, Y; Ishimatsu, H; Tabata, Y; Nishihara, T; Terashita, M. Formation of Dentin-like Particles in Dentin Defects above Exposed Pulp by Controlled Release of Fibroblast Growth Factor 2 from Gelatin Hydrogels. *J Endodont* 2007;33:1198-1202

[14] Ahmed, TA; Dare, EV; Hincke, M. Fibrin: a versatile scaffold for tissue engineering applications. *Tissue Eng* Part B 2008; 14:199-215.

[15] Marx, RE; Carlson, ER; Eichstaedt, RM; Schimmele, SR; Strauss, JE; Georgeff, KR. Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodon* 1998;85:638-646.

[16] Anitua, E. Plasma rich in growth factors: preliminary results of use in the preparation of sites for implants. *Int J Oral Maxillofac Implants* 1999;14:529-535.

[17] Anitua, E, Andía, I; Ardanza, B; Nurden, P; Nurden, AT. Autologous platelets as a source of proteins for healing and tissue regeneration. Thromb Haemost 2004;91:4-15.

[18] Nurden, AT; Nurden, P; Sánchez, M; Andía, I; Anitua, E. *Platelets and wound healing.* Frontiers Biosci 2008;13:3532-3548.

[19] Anitua E, Sánchez M, Orive G, Andía I. The potential impact of the preparation rich in growth factors (PRGF) in different medical fields. *Biomaterials* 2007;28:4551-4560.

[20] Anitua, E; Andia, I; Sanchez, M; Azofra, J; del Mar Zalduendo, M; de la Fuente, M; Nurden, P; Nurden, AT. Autologous preparations rich in growth factors promote proliferation and induce VEGF and HGF production by human tendon cells in culture. *J Orthop Res* 2005;23:281-286.

[21] Anitua, E. Enhancement of osseointegration by generating a dynamic implant surface. J *Oral Implantol* 2006;32:72-76.

[22] Hokugo, A; Ozeki, M; Kawakami, O; Sugimoto, K; Mushimoto, K; Morita, S; Tabata, Y. Augmented bone regeneration activity of platelet-rich plasma by biodegradable gelatin hydrogel. *Tissue Eng* 2007;11:1224-1233.

[23] Murray, MM; Spindler, KP; Abreu, E; Muller, JA, Nedder, A; Kelly, M; Frino, J; Zurakowski, D; Valenza, M; Snyder, BD; Connolly, SA. Collagen platelet rich plasma hydrogel enhances primary repair of the porcine anterior crucate ligament. *J Orthop Res* 2007;25:81-91.

[24] Intini, G; Andreana, S; Intini, FE; Buhite, RJ; Bobek, LA. Calcium sulphate and platelet rich plasma make a novel osteoinductive biomaterial for bone regeneration. *J Translational Med* 2007;7;5:13.

[25] Tsay, RC; Vo, J; Burke, A; Eisig, SB; Lu, HH; Landesberg, R. Differential growth factor retention by platelet rich plasma composites. *J Oral Maxillofac Surg* 2005;63:521-528

[26] Weibrich, G; Hansen, T; Kleis, W; Buch, R; Hitzler, WE. Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration. *Bone* 2004;34:665-671

[27] Schnabel, LV; Mohammed, HO; Miller, BJ; McDermott, WG; Jacobson, MS; Santangelo, KS; Fortier, LA. Platelet rich plasma (PRP) enhances anabolic gene

expression patterns in flexor digitorum superficialis tendons. *J Orthop Res* 2007;25:230-240.

[28] Ito, K; Yamada, Y; Nagasaka, T; Baba, H; Ueda, M. Osteogenic potential of injectable tissue-engineered bone: a comparison among autogenous bone, bone substitute (Bio-Oss®), platelet rich plasma, and tissue engineered bone with respect to their mechanical properties and histological findings. *J Biomed Mater Res* A 2005;73:63-72

[29] Yamada, Y; Ueda, M; Hibi, H; Nagasaka, T. Translational research for injectable tissue-engineered bone regeneration using mesenchymal stem cells and platelet rich plasma: form basic research to clinical case study. *Cell Transplant* 2004;13:343-355

[30] Kitoh, H; Kitakoji, T; Tsuchiya, H; Mitsuyama, H; Nakamura, H; Katoh, M; Ishiguro, N. Transplantation of marrow-derived mesenchymal stem cells and platelet rich plasma during distraction osteogenesis: a preliminary result of three cases. *Bone* 2004;35:892-898.

[31] Yamada, Y; Ueda, M, Hibi, H; Baba, S. A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: a clinical case report. *Int J Periodontics Restorative Dent* 2006;26:363-369.

[32] Anitua, E; Sanchez, M; Nurden, AT; Zalduendo, M; de la Fuente, M; Orive, G; Azofra, J; Andía, I. Autologous fibrin matrices: a potencial source of biological mediators that modulate tendon cell activities. *J Biomed Mat Res* 2006;77:285-293.

[33] Bielecki, TM; Gazdzik, TS; Arendt, J; Szczepanski, T; Krol, W; Wielkoszynski, T. antibacterial effect of autologous platelet gel enriched with growth factors and other active substances. *J Bone Joint Surg* 2007;89:417-420.

[34] Gruber, R; Varga, F; Fischer, MB; Watzek, G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. *Clin. Oral Implants Res.* 2002;13:529-535.

[35] Weibrich, G; Gnoth, SH; Otto, M; Reichert, TE; Wagner, W. S*timulation of the proliferation rate of human osteoblast like cells by platelet concentrates in vitro*. Mund. Kiefer Gesichtschir. 2002;6:168-174.

[36] Doucet, C; Ernou, I; Zhang, Y; Llense, JR; Begot, L; Holy, X; Lataillade, JJ. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol.* 2005;9999:1-9.

[37] Celotti, F; Colciago, A; Negri-Cesi, P; Pravettoni, A; Zaninetti, R; Sacchi, MC. Effect of platelet-rich plasma on migration and proliferation of SaOS-2 osteoblasts: role of platelet-derived growth factor and transforming growth factor-beta. *Wound Rep*. Reg. 2006;14:195-202.

[38] Ye, L; Peng, L; Tan, H; Zhou, X. HGF enhanced proliferation and differentiation of dental pulp cells. *J. Endod.* 2006;32:736-741.

[39] Anitua, E; Orive, G; Plá, R; Román, P; Serrano, V; Andía, I. The effects of PRGF on bone regeneration and on titanium osseointegration in goats: a histologic and histomorphometric study. *J Biomed Mater Res A* (In Press).

[40] Dean, JW; Culbertson, KC; D'Angelo, AM.. Fibronectin and laminin enhance gingival cell attachment to dental implant surfaces in vitro. *Int. J. Maxillofac. Implants* 1995;10:721-728.

[41] Schneider, G; Burridge, K. Formation of focal adhesions by osteoblasts adhering to different substrata. Exp. *Cell Res.* 1994;214:264-269.

[42] Anitua, E. Novel protocols for a predictable implantology. *Pract Proced Aesthet Dent* 2008; 20(2):123-128.

[43] Torrella, F; Pitarch, J; Cabanes, G; Anitua, E. Ultrasonic osteotomy for the surgical approach of the maxillary sinus: a technical note. *Int J Oral Maxillofac Implants* 1998;13:697-700.

[44] Lee, E; Anitua, E. Atraumatic ridge expansion and implant site preparation with motorized bone expanders. *Pract Proced Aesthet* 2006;18:17-22.

[45] Anitua, E; Orive, G; Aguirre, JJ; Ardanza, B; Andía, I. 5-year clinical experience with BTI dental implants: risk factors for implant failure. *J Clin Periodontology* (In press 2008).

[46] Anitua, E; Orive, G; Aguirre, JJ; Andía, I. 5 year clinical evaluation of short dental implants placed in posterior areas: a retrospective study. *J Periodontology* 2008; 79: 42-47. Anitua, E; Orive, G; Aguirre, JJ; Andía, I. Clinical outcome of immediately loaded BTI dental implants: a 5-year retrospective study. *J Periodontology* (In press 2008)

[47] Ruckley, CV. Socioeconomic impact of chronic venous insufficiency and leg ulcers. *Angiology* 1997;48:67-69.

[48] Diegelman, RF; Evans, MC. Wound healing: an overview of acute fibrotic and delayed healing. *Frontiers in Bioscience* 2004;9:283-289.

[49] Anitua, E; Aguirre, JJ; Algorta, J; Ayerdi, E; Cabezas, AI; Orive, G; Andia, I. Effectiveness of autologous preparation rich in growth factors for the treatment of chronic cutaneous ulcers. *J Biomed Mater Res* B 2008;84:415-421.

[50] Sánchez, M; Anitua, E; Azofra, J; Andía, I; Padilla, S; Mujika, I. Comparison of surgically repaired achilles tendon tears using platelet–rich fibrin matrices. *Am J Sports Med* 2007;35:245-251.

[51] Sanchéz, M; Azofra, J; Anitua, E; Andía, I; Padilla, S; Santisteban, J; Mujika, I. Plasma rich in growth factors to treat an articular cartilage Avulsion: A Case Report. *Med Sci Sports Exer* 2003;35:1648-1652.

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Chapter 8

COMPARISON OF THE EFFECT OF FOUR NON-METAL POSTS ON FRACTURE RESISTANCE OF ENDODONTICALLY TREATED TEETH

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ABSTRACT

Statement of problem: The fracture resistance of the root canal is mainly associated to the kind of post used for treatment, and studies indicate that there are still different opinions about the fracture resistance of root canals when non-metal posts are used.

Purpose: The purpose of this study was to compare the fracture resistance of four adhesive non-metal posts with different MOEs against compressive load in endodontically treated teeth.

Materials and Methods: In this in vitro experimental study a total of 40 recently extracted mandibular premolars were selected, sectioned adjacent to the CEJ, and endodontically treated. The specimens were randomly assigned to four groups (n=10). Post spaces were prepared and the fiber D. T. Light posts, D. T. Composi post, D. T. White posts and Cosmopost were cemented. Composite resin (Lumiglass) cores were built up. After simulating the PDL the specimens were embedded in acrylic resin, and then secured in a Universal Testing Machine. A compressive load was applied at a 45° angle to the long axis of the tooth until fracture, at a crosshead speed of 1mm/min. Data were analyzed using (one-way) ANOVA and Tukey's test ($p<0.05$).

Results: Teeth restored with D. T. White fiber posts exhibited significantly higher resistance to fracture. Teeth restored with the other three groups were statistically similar ($p>0.05$). Fractures observed in fiber groups would allow further restorations of the tooth, whereas unrestorable, catastrophic fractures were observed in the zirconia group.

Conclusion: Zirconia post, due to the unfavorable and unrestorable fractures caused, should be used with more consideration and therefore indication of fiber posts is suggested.

Key words: fiber posts, zirconia posts, fracture resistance

INTRODUCTION

Roots' resistance to fracture is one of the most important factors in selecting the best restorative alternative for endodontically treated teeth with extensive loss of tooth structure. Posts are recommended to strengthen weakened endodontically treated teeth against intraoral forces by distributing torquing forces along the roots [1]. Despite the various attempts that have been made, vertical root fractures of pulpless teeth are still encountered in everyday clinical practice. It is well known that the use of posts to restore endodontically treated teeth does not increase the strength of the remaining tooth. Also preparing the post space increases the risk of micro fractures and perforation during the procedure [2].

Many studies have shown that pulpless teeth without post space preparation have a better resistance against occlusal forces than teeth with intra canal post space preparation [3]. Therefore a post and core system should only be considered when no other alternative is possible to provide retention and support for the restoration of teeth lacking coronal tooth structure [3]. Cast metal post and core foundations have superior physical properties however when retreatment of the root canal is necessary, removal of the post without further damage to the tooth structure is difficult. In recent years, various types of fiber composite posts such as carbon fiber, glass fiber, quartz fiber and their combination also known as the third generation of fiber composite posts have been introduced to the market. Fiber composite posts due to their greater tensile strength and similar MOE to dentin are preferred over cast metal posts. They allow a more conservative post space preparation, are not affected by corrosion, and bond with the Bis-GMA resin cement, therefore strengthen the structure of the remaining tooth while distributing the forces evenly throughout the tooth. Also if access to the root canal is required, they can be easily removed using drills [4]. Zirconia posts were introduced in the late 1980s and exhibited high fracture resistance and also had the ability to bond with resin cements [5] [6]. It has been reported that restored teeth with fiber posts have a higher resistance to fracture compared to teeth restored with zirconia posts [4]. But other studies have concluded that resistance to fracture of restored teeth with zirconia posts is equivalent to fiber posts [7] [8].

Rosentritt et al compared the fracture strength of endodontically treated teeth restored with titanium, zirconia, and fiber-reinforced posts and concluded that the fracture strength of teeth restored with zirconia posts was superior to that of titanium posts and both were superior to fiber-reinforced posts [9]. In a study by Mannocci et al intermittent loading of teeth restored with quartz fiber, carbon-quartz fiber, and zirconia posts was evaluated. Fiber-reinforced posts were able to reduce to a minimum the risk of root fractures and displayed significantly higher survival rates than teeth restored with zirconia posts [10]. Another study investigated stiffness of zirconia, titanium, and carbon fiber posts and concluded that zirconia posts exhibited superior characteristics [11].

Numerous studies have reported unfavorable and unrestorable fractures caused by Zirconia posts [12] [13] [14]. According to the results of butz et al, the fracture strength of teeth restored with zirconia posts and composite cores were significantly lower than those restored with zirconia posts and heat-pressed ceramic cores [15] [16].

There is limited information in the literature on comparison of post systems with different modulus of elasticity and their effect on the fracture resistance of root canal treated teeth. This study was conducted to compare the fracture resistance and mode of fracture of

endodontically treated teeth restored with non-metallic Zirconia, Quartz fiber, Translucent Quartz fiber and Carbon fiber posts against compressive load at the department of fix prosthetic dentistry of the Azad dental university of Tehran.

MATERIALS AND METHODS

In this in vitro study, a total of forty freshly extracted maxillary canines free of cracks, caries, and fractures were selected. All external debris was removed with an ultrasonic scaler, and the teeth were stored in saline-solution when not under testing.

A Caliper was used to selected teeth with the following dimensions: mean length of 21.5 mm with 14mm of root length, 5.5 to 6 mm mesiodistal and 7.0 to 8.0 mm faciolingual. It was determined that after the preparation of the post space, a minimum thickness of 1.5mm dentin must remain around the post space to eliminate changes caused by morphological or dimensional factors.

All specimens were observed with a stereo microscope at a two times magnification to insure that no root fractures exist. All the samples were stored in 37°c saline-solution till the day of the experiment.

The anatomic crowns of all teeth were sectioned horizontal to the long axis, at the cement-enamel junction (CEJ), with the use of a high speed water-cooled disk [17].

The working length of all canals was established with radiography, and all specimens were prepared to a size of 35 file (K-file 25mm, Densply, Mailefer) with consecutive filing and irrigation (%5.25 sodium hypo chloride), and size 35 file was selected as the master apical file (MAF). The specimens then were stepback prepared to a size of 60 file.

The canals were dried with paper points, and the roots were obturated with lateral condensation of size 15 gutta-percha and AH26 eugenol free sealer (Densply, Mailefer). The master gutta-percha point was coated with sealer and seated in the canal to the full working length. A finger spreader was inserted into the canal to a level approximately 1mm short of the working length.

After all of the above preparations the teeth were randomly assigned to 4 groups of 10 teeth each [18] [19].

Restorative materials used for posts and core build-up are listed in table I.

Table I. Restorative materials used for posts and core build-up

Materials	Manufacturers	Post Modulus of elasticity/(GPa)
D.T. LIGHT Post (translucent quartz fiber [glass fiber])	RTD (France)	14
D.T. WHITE Post (quartz fiber)	RTD (France)	15
D.T. Composi post (carbon fiber)	RTD (France)	21
Cosmopost (zirconium oxide)	Ivoclar- vivadent (Lichtenstein)	210
Adhesive system (dual cure)	Panavia F2.0 (Japan)	_
Resin cement (dual cure)	Panavia F2.0 (Japan)	_
Composite Core Build up (light cure)	Lumiglass, RTD, (France)	_
AH – 26 Sealer	Densply, Maillefer (Switzerland)	_

To prepare the post space peeso reamers size 1, 2 and 3 and later the drills from the universal drill kit were used. Post space preparation was terminated at 10mm length, and to ensure a good seal, 3–5mm was kept in the apex. Afterwards a special finishing drill in size 3 was used for the final preparation. (Silicone stoppers were placed around the drill shaft to control the working length.)

Enlarged canals were rinsed with water spray and silane, and dried with paper cones and stream of air. Posts were placed into prepared canals to insure passive fitting.

All posts were sectioned horizontally with a water-cooled diamond fissure bur with a length of 15mm [20].

Post space was dried using paper points, then the root canal walls and core sitting place were etched with 37% phosphoric acid for 15 seconds, washed with water sprat, and gently air dried (3 seconds) to avoid dehydration of dentin.

Primer A & B were mixed according to the manufacturer's recommendations, then brushed on the intra-canal and cervical dentin with a disposable brush. After 30 seconds the primer was dried with paper cones and stream of air.

Equal measures of A & B catalyst and base pastes (Panavia F2.0) were hand mixed. The D.T. Light, D.T. White, and D.T. Composi posts with a 1.2mm diameter and Cosmopost with 1.4mm diameter were coated with the mixture and inserted in to the canals and seated with finger pressure for 5–10 seconds. Excess resin cement was removed with a disposable brush; the cement was then light cured for 60 seconds. Complete setting of the cement was accomplished by using Oxy-guard around the insertion area for three minutes.

The intensity of the light was monitored with a curing radiometer, and it was in excess of 400 mw/cm2 throughout the study.

The crown portion of the posts were coated with lutting adhesive, then previously made polyester premolar-shaped matrices were filled with composite (Lumiglass), and seated over the posts crown portion. The specimens were light-cured for 40 seconds in four different directions (facial, lingual, mesial, and distal). The preformed polyester matrix was then removed with a surgical blade.

Later to simulate periodontal ligament, root surfaces were root planned and marked 1–2 mm below the CEJ and covered with 0.2 mm triangular shapped adapta foils from the marked place to the apical end to obtain a uniform thickness. Specimens then were embedded in autopolymerizing acrylic resin surrounded by alluminium cylinders. After the first sign of polymerization, teeth were extracted from the resin blocks by moving rods in an upward direction, and Adapta spacers were removed from the root surfaces. Poly ether (Impergum/ESPE) impression material was injected into acrylic mold, and the teeth were reinserted into resin cylinders. The extruded elastic impression material around the CEJ was then removed with a surgical blade after complete setting [21] [22].

The upper level of the acrylic resin was considered 1.5–2 mm below the CEJ.

The specimens were kept in silane solution at room temperature during the rest of the experiment.

Specimens were secured in a universal load-testing machine (Instron 1195). Compressive load was applied to the lingual inclination of the buccal cusp of the core at a 45-degree angle to the long axis of the specimens (to simulate intra oral forces of mastication) at a crosshead speed of 1mm/min until fracture occurred. The fracture loads were determined, and the mode of fracture was recorded and classified as favorable (meaning that the fractures were not

below the cervical area, and therefore are easily repaired using crown lengthening procedures) or unfavorable (oblique and more than 1–2mm below the cervical area).

RESULTS

The mean failure values and SDs anre presented in table 2. One-way ANOVA and One-sample kolmogorov-smirnov tests were conducted for statistical evaluation and deviation from normal distribution ($P<0.05$).

Table II. The mean failure values and SDs in tested groups

Group	(n)	Mean Fracture Strength	SD	SD Error	95% Confidence Interval for Mean		Min	Max
					Lower Limit	Upper Limit		
D.T. Light	10	668.8889	198.64821	66.21607	516.1944	821.5834	390.00	895.00
D.T. White	10	917.7778	167.39010	55.79670	789.1104	1046.4452	700.00	1180.00
D.T. Composi	10	628.7778	187.36847	62.45616	538.7536	826.8019	400.00	920.00
Cosmopost	10	819.4444	128.41448	42.80483	720.7363	918.1525	520.00	945.00

As shown in table 2, the highest fracture resistance was recorded for D.T. White posts. The results from an One-way ANOVA test revealed significant differences between the groups ($p<0.05$)

For a more conclusive analysis and pair wise comparison among groups, Tukey HSD test was conducted (Table III).

The results were as followed:

- Difference in fractures resistance of D.T. White and Zirconia groups are not significant ($p=0.438$).
- Difference in fractures resistance of D.T. Light and Zirconia groups are not significant ($p=0.158$).
- Difference in fractures resistance of D.T.Light and D.T.White groups are significant, therefore D.T.White posts have a greater resistance to fracture compared to D.T.Light posts ($p=0.011$).
- Difference in fractures resistance of D.T.Composi and D.T.Light groups are not significant ($p=0.998$).
- Difference in fractures resistance of D.T.Composi and D.T.White groups are significant, therefore D.T.White posts have a greater resistance to fracture compared to D.T.Composi posts ($p=0.033$).
- Difference in fractures resistance of D.T.Composi and Zirconia groups are not significant ($p=0.350$).

Table III. Tukey HSD test for pair wise comparison among groups, multiple comparisons

(I) GROUP	(J) GROUP	Mean Difference (I-J)	SD Error	Sig.	95% Confidence Interval for Mean	
					Upper Limit	Lower Limit
glass fiber	quartz fiber	-248.8889(*)	81.33531	.022	-28.5221	-469.2557
	zirconia	-150.5556	81.33531	.269	69.8112	-370.9223
	carbon fiber	-13.8889	81.33531	.998	206.4779	-234.2557
quartz fiber	glass fiber	248.8889(*)	81.33531	.022	469.2557	28.5221
	zirconia	98.3333	81.33531	.626	318.7001	-122.0334
	carbon fiber	235.0000(*)	81.33531	.033	455.3668	14.6332
zirconia	glass fiber	150.5556	81.33531	.269	370.9223	-69.8112
	quartz fiber	-98.3333	81.33531	.626	122.0334	-318.7001
	carbon fiber	136.6667	81.33531	.350	357.0334	-83.7001
carbon fiber	glass fiber	13.8889	81.33531	.998	234.2557	-206.4779
	quartz fiber	-235.0000(*)	81.33531	.033	-14.6332	-455.3668
	zirconia	-136.6667	81.33531	.350	83.7001	-357.0334

* The mean difference is significant at the .05 level.

Table IV display the percentage and different fracture types observed in each group.

Table IV. Percentage and different fracture types observed in each group.

Fracture type / Group	Core separation	Core fracture	Root fracture in cervical area	Oblique root fracture	Post & core separation	Vertical root fracture	Post fracture
D.T. Light	%44	%55	%100	—	%11	—	—
D.T. White	%11	%88	%100	—	%22	—	—
D.T. Composi	%33	%55	%100	—	%11	—	—
Cosmopost	—	%100	%77	%22	—	—	%100

It should be noted that the line of fracture in fiber groups were mainly observed in the core and %100 of the fractures were favorable and restorable, but the Zirconia group exhibited %55 unfavorable fractures and only %45 favorable fractures.

Diagram I demonstrates minimum, mean and maximum resistance to fracture (N) of specimens for each group.

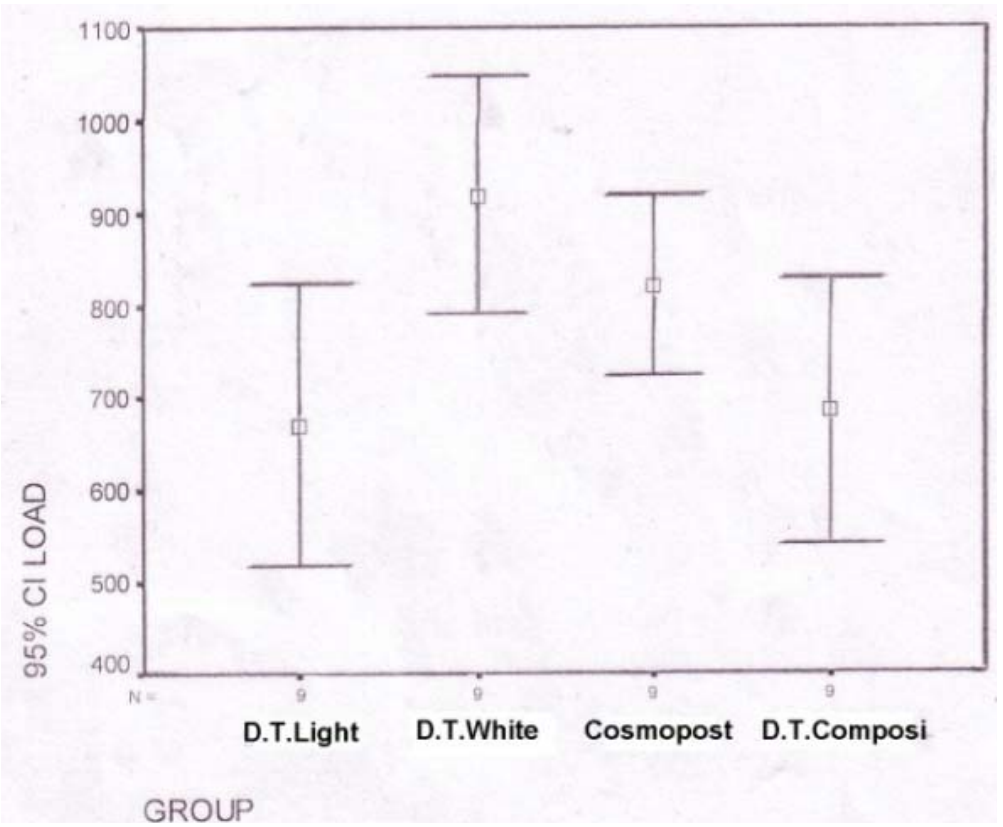

Diagram I. Minimum, mean and maximum resistance to fracture (N) of specimens for each group.

CONCLUSION

Several factors play an important role in proper selection of a prefabricated esthetic post: biomechanical and physical properties, shape, esthetic, cost, technique sensitivity [18]. There are a variety of posts with different compositions in the market that meet the requirements for these factors.

This study compared the resistance to fracture of teeth restored with four different non-metallic post systems.

The first group was restored with D.T. Light posts, the second group with D.T. White, the third group with D.T Composi and the fourth group was restored with Zirconia posts (cosmopost).

The results indicated that the group two specimens (D.T.White) demonstrated the highest mean resistance to fracture, while there was no significant resistance to fracture between the other three groups. But the resistance to fracture recorded from all four groups was higher than the normal intraoral forces therefore the use of all four types of posts is recommended for the treatment of pulpless teeth.

Group 4 (Zirconia) exhibited the highest percentage of unfavorable fractures which results from the rigidity and higher MOE of Zirconia posts compared to dentin, also removing the fractured post particles from the canal is more challenging.

But statistical analysis of the mode of fracture showed that the fiber groups fractured favorably. This is due to the similar MOE of the posts with dentin that enables better distribution of stress along the restored tooth.

Studies that compared the mechanical properties of different post systems suggest that the ability of each post-and-core system to protect the root from biomechanical failure may vary greatly [7][10][11][15].

Meaningful comparison with results of other similar studies of in vitro fracture resistance is impossible because of the number of variable involved. Such variables include tooth conditions previous to the extraction, tooth age, tooth storage conditions, pulp situation at the time of extraction, root anatomy and dimensions, and loading angle and location [18].

In the present research particular care to standardize the root dimensions has been taken.

Proper consistency of poly-ether impression material was used to simulate the periodontal ligament surrounding the tooth to allow natural movement [7]. When testing without the artificial ligament, the acrylic resin acts as a ferrule, which has a significant effect in preventing root fracture, and recording higher resistance to fracture [17]. Therefore, in the current situation, the usage of artificial ligament was the most appropriate method, even though it was not a natural one.

In this study, the post-supported cores were not restored with crowns, as in similar previous studies, the compressive load was directly applied to the inclined surfaces of the cores at a 45-degree angle [1]. In this manner, the probable altering of parameters, such as material structure, shape, length, and thickness, by crown restorations was avoided. It is considered that by eliminating such parameters, the structural integrity and fracture resistance of a post-and-core foundation could be tested more precisely [1]. Therefore the entire external load is transmitted to the core, while Inclusion of the final crown would have transmitted part of the load directly to the root. A similar method was used in studies conducted by Taner [1] and Barjua [20].

The posts used in this study were provided by different manufacturers and therefore differ in diameter. But great care was made to select posts with nearest diameter.

The manufacturers of fiber posts claim that the slightly double-tapered posts, which closely resemble the morphology of the root form, absorb stress rather than transferring it and thus protect endodontically treated teeth against fracture [7].

In this study a 45 degree loading to the long axis of the tooth was applied to simulate the average functional angle of normal occlusion [17].

It was previously reported that the adhesive luting agent had a significant strengthening effect on the post retention within resinous core materials. Probable voids or bubbles within the core or gaps at the post-core interface resulting from a lack of condensation when placing the resin material around the posts may affect the integrity of the post-and-core foundation, and consequently, the compressive force resistance of the post-and-core foundation. To enhance the bond between the post heads and the cores, adhesive resin luting agents can be applied to the post prior to forming the core [1] [23]. The same method was carried out in this study.

In the present study we concluded that resistance to fracture of teeth restored with D.T.White posts is higher than Zirconia, D.T.Light, D.T.Composi which can be due to the

favorable condition and unity of this post with the luting cement and bonding agent, and also the closer MOE of this post to dentin. Also the fiber groups fractured favorably, whereas Zirconia fractures were mainly unfavorable.

Akayan et al. [7] reported that Quartz fiber posts compared to Glass fiber, Zirconia and Titanium post have a higher resistance to fracture and the fractures in the Titanium and Zirconia posts are unfavorable and catastrophic but the fracture pattern in the fiber post is restorable which are in agreement with findings in the present study.

No significant difference was recorded when comparing the resistance to fracture of D.T.Light and D.T.Composi, although resistance to fracture of Zirconia group was higher than the Glass fiber, which can be due to the stronger bond of Zirconia with the luting cement [5]. Cormier [21] and Rosentrit [9] reported similar findings.

Also Mannocci et al. [10] reported that Quartz fiber posts can minimize the risk of root fractures, which is in agreement with the results of our study.

But our results differ from those concluded by Maccari and his colleges [18], where they stated that teeth restored with Zirconia have a smaller resistance to fracture compared with Glass and Carbon fiber posts. A possibility to explain the difference could be the use of different bonding agents and adhesive cements that could create a better seal and unity with the Zirconia post and therefore recording a higher resistance to fracture. The perfect fit of the post with the prepared space can also be noted. However they too experienced unfavorable fracture with Zirconia posts.

Zirconia posts exhibited unfavorable fractures which are in agreement with the findings of Maccari [18], Taner [1], Akkayan [7], Mitsui FH [12] and Cormier [21].

In this respect, many studies have indicated that ceramic posts are rigid and induce more stress to the root compared with fiber posts and therefore causing unfavorable damage to the root [11][13][14].

In the present study Zirconia fractures were all brittle and the fractured particles remained in the canal, which were also experienced by Cormier [21], Maccari [18] and Akkayan [7]. Shahmali & Demirel [6] and Taner [1] also noted that the bond between zirconia and cement is very strong.

The fact that removing fractured ceramic particles from the canal of a failed restoration is difficult, counts as major clinical disadvantage [1] [7] [18].

The mode of failure in fiber posts is 100% favorable. Similar findings were reported by Hayashi [17], Akkayan [7], Barjua [20] and Mitsuie [12].

The different modes of failure observed among the groups can be explained by the difference in the modulus of elasticity of material [7] [17] [18].

The replacement and reinforcement of intraradicular tooth structure with a material having an elastic modulus similar to that of dentin is better than replacing lost intraradicular tooth structure with an inflexible material [1]. When a post-core with a high modulus of elasticity, such as Zirconia (around 170 GPa) [11], is forced against radicular dentin with much lower modulus (around 18.6 GPa) [7], the stress is transferred from the rigid post to the less rigid dentin. When a post with a similar modulus of elasticity to that of radicular dentin, such fiber post, is used for restoration, less stress is transferred from the post to the dentin [19].

Regarding core fractures, D.T.White group in 88%, D.T.Light and D.T.Composi in 55%, and 100% of the Zirconia group exhibited fractures which count as a disadvantage for these systems. Therefore it is advised that the use of such systems in high stress bearing prosthesis

should be with more consideration. To prevent this problem, increasing the strength of the composite core using highly filled composites with a higher compressive strength can be indicated [24].

It was previously reported that when a Zirconia post is used with a direct composite core, large stress bearing composite cores in combination with subgingival margins should be avoided. Zirconia posts with heat-pressed ceramic cores have been suggested for use because their similar thermal expansion coefficients may result in favorable shrinkage and fit of the restoration [9][15][16]. In present study, Zirconia posts with a flexural strength of 820 Mpa were used with composite cores. This may account for the fracture under compressive load of all teeth restored with Zirconia posts. A similar result was reported by Butz et al. [16] and Akayan [7].

It has been reported that the ordinary chewing force of adults ranges from 400-800(N) [15][22]. The fracture loads in all groups were found to be sufficiently greater than the ordinary chewing force (668.8(N) for D.T. Light, 917.7(N) for D.T. White, 682.7(N) for D.T. Composi and 819.4(N) for Zirconia).

CONCLUSION

According to the results of the present study it can be concluded that teeth restored with these non-metallic posts exhibit a higher resistance to root fracture against intra oral forces, but in this respect, Quartz fiber posts (D.T. White) compared to the other three groups demonstrated resistance to root fracture to greater forces. Although Zirconia posts can provide esthetical needs, due to their high MOE a high incidence of catastrophic (non-restorable) fractures and the fact that removing the remains of a fractured Zirconia post to initiate another restoration might become an insurmountable clinical problem, the use of fiber posts is preferred.

SUGGESTIONS

The use of the new adhesive technology to enhance the structural integrity between different post-and-core materials, and their fracture resistance is being considered for future research.

Future research should focus on how the length, size and design of the post, the cementing technique or the post insertion parameters influence the biomechanics of restored teeth, thus insuring a more robust restoration technique. These analyses will allow data that can help to reduce post-treatment iatrogenic lesions that could result.

REFERENCES

[1] Faruk Taner Dilmener, DDS, PHD, Cumbur Sipani. DDS, PhD, and Mehmet DalKiz, DDS, PhD. Resistance of three new esthetic post- and core systems to compressive loading. *J prosthet Dent 2006; 95:130-6.*

[2] Koutayas So, Kern M,Ferraresso F, Stub JR. Influence of design and mode of loading on the fracture: An In vitro study in a dual axis chewing Simulator.*J Prosthet Den 2000; 83: 540-547.*

[3] Davide Assif, DMD and Colin Grofil, BDS. Biomechanical consideration in Restorating endodontically treated teeth. *J prosthet dent 1994; 71: 565-7.*

[4] A, Peutzfeldt A, Asmussen E, Gotfredsen K. Bond Strength of resin cement to dentin and to surface – treated posts of titanium alloy, glass fiber and zirconia. *J adhes Dent (2003); 5(2) : 153-62.*

[5] Fokkinga wa, Kreulen Cm: A structured analysis of in vitro failure loads and failure modes of fiber, metal, ceramic posts and core systems: *J Prosthet Dent 2004- 17 (4) 476- 482.*

[6] Shahmali S, Demirel F: Comparison of in vitro tensile bond strengths of luting cement to metallic and tooth colored posts: *J Periodontics restorative dent. 2004; 24 (3) 256-263.*

[7] Begum Akkayan, Dr Med Dent, and Turgut Gulmez, PHD. Resistance to fracture of endodontically treated teeth restored with different post systems. *J of prosthetic dentistry 2002 :431-437.*

[8] Stockton LW, Williams PT. Retention and shear bond strength of two post systems. *Oper Dent (1999) : 24(4) : 210 – 6.*

[9] Rosentrit M.Furer C. Behr M, Lang R, Handel G. Comparison of In vitro Fracture strength of metallic and tooth coloured posts and Cores. *J oral Rehabil 2000; 27: 595.601.*

[10] Mannocci F, Ferrari M, Watson TF. Intermittent Loading of teeth restored using quartz Fiber, carbon-quartz Fiber, and zirconium dioxide ceramic root canal posts. *J Adhes Dent 1999: 1; 153-8.*

[11] Asmussen E, Peutzfeldt. A. Heitmann T. Stiffness, elastic limit and strength of newer types of endodontic posts. *J Dent 1999; 27: 275-8.*

[12] Mitsui Fh; Invitro study of fracture resistance of bovine roots using different intraradicular post system, *Quintessence Int, 2004- 35 (8) 612 – 616.*

[13] Dietschi D , Romelli M , Goretti A. Adaptation of adhesive posts and cores to dentin after fatigue testing. *Int J prosthodont 1997; 10: 498-507.*

[14] Purton DG, Love RM, Chandler NP. Rigidity and retention of Ceramic root canal posts. *Oper Dent 2000; 25: 223-227.*

[15] Assif D, Gorfi, C. Biomechanical consideration in restoring endodontically treated teeth. *J Prosthet Dent 1994, 71: 565.*

[16] Butz F, Lennon AM , Heydecke G, Stub JR. Survival rate and fracture strength of endodontically treated Maxillaly incisors with moderate defects restored with different post -and -core systems. *Int prosthodent, 2001 , 14: 58-64.*

[17] Mikako Hayashi, Yutaka Takahashi, Satoshi ImaZato, Shigeyuki Ebisu. Fracture resistance of pulpless teeth restored with post-cores and crowns. *Dental Materials (2006) 22(5), 477 – 485.*

[18] Maccari PC,.Conceicao EN , Nunes MF. Fracture Resistance of Endodontically Treated teeth Restored with three different prefabricated esthetic posts. *J Esthet Restor Dent 15: 25-30, 2003.*

[19] Balbosh A, Kern M. Effect of surface treatment on retention of glass fiber endodontic Posts. *J prosthet Dent (2006), 95(3) 218-23.*

[20] Barjau – Escribano A, Sancho – Bru JL, Forner – Navaroo, Rodriguez – Cervantes PJ, perez – Gonzalez A, Sanchez – Marin FT. Influence of prefabricated post material on restored teeth: Fracture strength and stress distribution. *Operative Dentistry, 2006; 31-1, 47-54.*

[21] Clarence J. Cormier, DDS, David R. Burns, DMD, and peter Moon. PhD. In vitro comparison of the Fracture resistance and failure mode of fiber, ceramic and conventional post systems at various stages of restoration. *J of prosthodontics, 2001: p 26-36.*

[22] Erfani S, Jalalian E. *Invitro study on the shear strength of anterior and posterior FRC Bridges*. Tehran Azad School of Dentistry. Final paper for doctoral degree. (2006-2007); 260-7.

[23] Aksoy G. Cotert HS, korkut L. Effect of an adhesive resin luting agent on the dowel – head retention of three different core materials. *J Prothet Dent 2005; 93: 439-45.*

[24] Stephen C. Bayne, Jeffery Y. Thompson, Duane F. Taylor. Dental Material. In: Theodore M. Roberson, Harald O. Heymann, Edward J. Swift, JR, Editors. *The Art and Science of Operative Dentistry. Fouth edition. Mosby (2002) p.133-234*

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Short Commentary 1

DIFFERENCES IN DENTIN PERMEABILITY AND ITS RELEVANCE TO CYTOTOXICITY

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ABSTRACT

In vitro cytotoxicity studies have gained tremendously in popularity in recent years. To make these in vitro studies more clinically relevant, diffusion experiments in an in vitro pulp chamber device (IVPC) using dentin has repeatedly been advocated. The purpose of this study was to investigate dentin permeability of different thicknesses at an anatomical position of the tooth and then to determine the dentin thickness necessary to mask the cytotoxicity of a recent toxic dentin bonding agent (Xeno III).

Materials and Methods: Molar teeth were cut just coronal of the pulp horns creating discs of different thicknesses. These discs (99) were tested for their permeability in an In Vitro Pulp Chamber (IVPC) device. Furthermore, near confluent mouse 3T3 cells were exposed to Dulbecco modified eagle's medium with extractions from Xeno III through dentin discs of different thicknesses (35) as well as through standard membranes. The cell viability (survival rate) was measured using the MTT test (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and related to the non-exposed controls.

Results: When the relationship of disc thickness and permeability was studied, a substantial variation in the results at a specified thickness for different subjects was found but statistical analyses demonstrated a significant link between thickness and permeability. The decrease in dentin permeability became really evident at a thickness of more than ~350 μm.In the cytotoxicity experiments it was found that for the artificial membrane group as well as for etched dentin discs with a thickness of betrween 120–200 μm, cell viability was around 25% compared to the control which was 100%. When cytotoxicity was evaluated for the 500 μm disc as barrier, cell viability was at 60.7% with a cell death of 39%. In the 750μm group, cell viability was much higher at 75% but still

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cell death was noticed when compared to the control. Only at a disc thickness of 1000 µm and above no cell death was observed.

Conclusion: Dentin permeability can not be predicted but should be determined separately for each disc as large variations between individual dentin discs were evident. Dentin permeability does not increase in a linear fashion with dentin thickness but rather exponentially. An alternative to dentin discs would be to choose a standard permeable membrane through which cytotoxicity can be measured. Furthermore, it cannot be assumed that no cytotoxicity will take place when using dentin barriers of 500 µm or even 750 µm. Xeno III should only be used in cavities with a remaining dentin thickness of at least 1000 µm between the cavity floor and the pulp.

INTRODUCTION

The placing of potentially cytotoxic materials like dentin bonding agents (DBA) or composite restorations close to the pulp is a common practice in dentistry and is gaining popularity due to esthetic requirements (Szep et al., 2002). As these materials have contact with various aqueous media in vivo which may extract leachable components, the biocompatibility of these materials is of utmost importance (Geurtsen et al., 1999). The optimal way to test biocompatibility would be by in vivo tests where the system is used under clinical conditions which, however, is seldom possible. As a result in vitro cytotoxicity studies have gained popularity in recent years as they do not make use of animal testing which has become increasingly unacceptable in the public mind. This has recently led to considerable interest being focused on cytotoxicity assays using cell culture to evaluate toxicity of materials.

It was consistently demonstrated that dentin is an effective diffusion barrier, preventing/limiting pulp damage not only from toxic substances such as eugenol and phenol, but also from HEMA (Schmalz et al., 2002). Hanks et al. (1988) showed that dentin can reduce the toxicity of resins by limiting diffusion of those substances from the cavity preparation to the pulp cavity. Dentin probably absorbs substances in the tubules and also limits the traverse of substances through the dentin (Hanks et al., 1994, Pashley 1988). According to Pashley et al 1988, the main reason for the decreased cytotoxicity when using dentin discs is due to the large internal surface area of dentin, which binds and traps a variety of substances. Furthermore, the barrier effect of dentin was clearly shown when dentin discs of increasing thickness reduced toxicity (Hanks et al., 1988 and Schmalz, 1994).

The choice of cell line for in vitro cytotoxicity screening assays however remains controversial, as the apparent cytotoxicity of a material can be affected by the cell line. However, use of permanent cell lines provides good reproducibility for in vitro cytotoxicity screening (Murray et al., 2000; Schmalz, 1988). Therefore, the purpose of this study was to investigate dentin permeability of different thicknesses at an anatomical position of the tooth and then to determine the dentin thickness necessary to mask the cytotoxicity of a recent toxic dentin bonding agent.

MATERIALS AND METHODS

Freshly extracted, intact human third molars were used for this study. The teeth were stored at 8ºC with a few crystals of thymol in water to prevent microbial growth. They were then cast in clear resin and ground with a grinding disc (50 grid), using water cooling, from the apical side until only the part of the crown remained that showed the pulp horns. Each tooth was then further ground with 220 grid sand paper and water (Chen et al., 2003) up to the point where the pulp horns were no longer visible. The remaining tooth was then sectioned just coronal of the pulp horns (Figure 1) using constant water cooling, by a 350 µm thick slow speed diamond disk blade (Strues Minitom Diamond cut off wheel) and ground on the coronal side until the desired thickness (measured with a Mitutoyo digital measuring device) was achieved. In the permeability experiments ~125 discs were prepared. Of these, 9 were for the ~120 µm, 9 for the ~200 µm, 8 for the ~350 µm and 73 for the ~500 µm disc group. The other discs were discarded for various reasons: like parts of enamel in dentin disc, a too small disc diameter, perforated dentin etc. To remove the smear layer completely the discs were treated with 10% citric acid and 3% $FeCl_3$ for 60 seconds (Cao et al., 1992). To stop the etching process the discs were vigorously washed in three separate glasses containing distilled H_2O. Subsequently, the hydrated discs were placed in the special pulp chamber devices (Figure 2; 20ml Micro reaction vessels by Supelco) between two rubber washers, also supplied by Supelco. Inc., (similar to Schmalz et al., 2001). The hydrostatic pressure of a 30 cm water column was applied to one side of the dentin disc. A capillary with a known diameter was placed between the manometer column and the inlet. The movement of an air bubble in a capillary system was measured against time, where time was measured taken to allow the diffusion of 5 µl through the standard opening on the dentin disc. The time the air bubble takes to move from one point to another represents the volume moving through the dentin by hydraulic conductance in that period of time. The temperature was kept constant at 23±1° C.

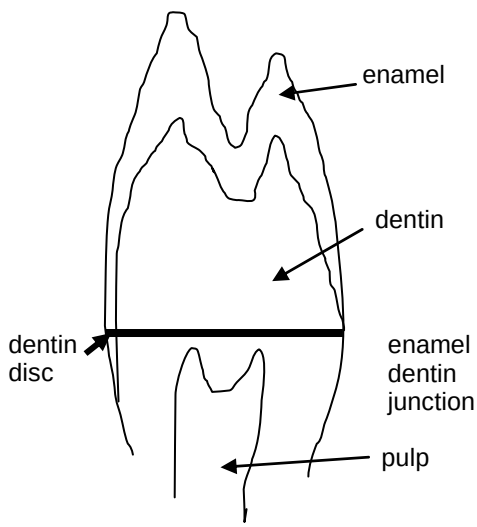

Figure 1. Origin position of dentin discs.

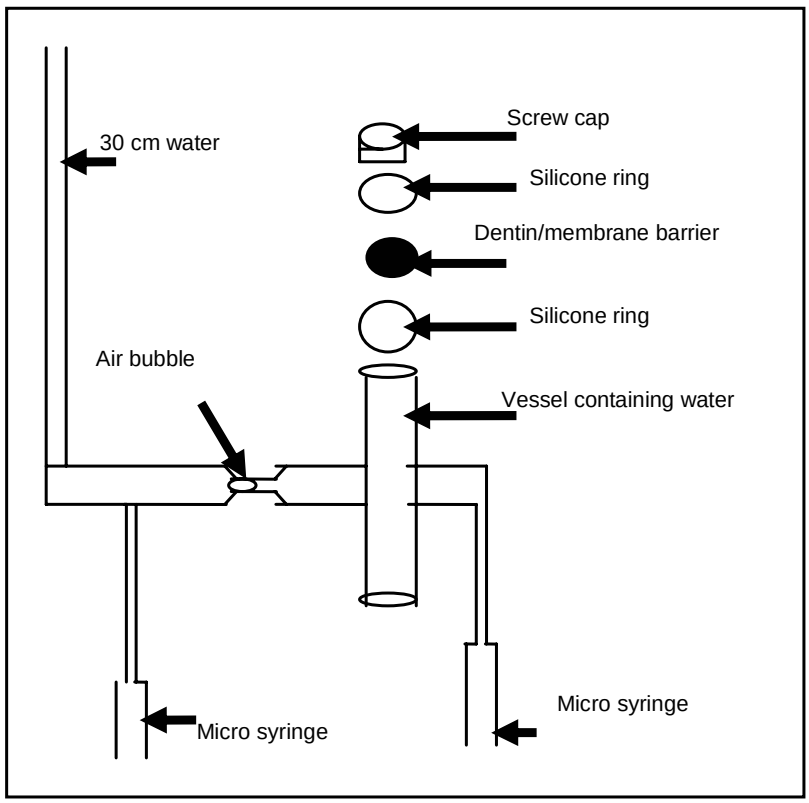

Figure 2. Diagram of apparatus (pulp chamber device) used to measure permeability.

For the cytotoxicity testing of the bonding agent, an adapted and indirect test model (Grobler et al., 1996; Grobler et al., 2008) was used. Briefly, the model (Figure 3) consisted of a sample bottle with an 8-mm hole in the screw top. Membrane discs (Omonics Micron Sep, Cellulosic, White, Plain, 45 micron) (or dentin discs of different thicknesses) were then placed inside the screw top as barrier. Dentin discs of ~120µm, ~500µm, ~750µm and ~1000µm where used as a barrier in extract tests. Eight µl of the self etching cytotoxic dentin bonding agent, Xeno III (Dentsply De Trey) was then applied to the outside of the disc and treated according to the manufacturer's instructions. Seven sample bottles were prepared for each of the above mentioned dentin thicknesses, 7 for the artificial membrane as well as 7 for the controls. The exact procedure was published (Grobler et al., 2008) and the diagramme (Figure 4) included for clarity. Each sample bottle was filled with 400 µl of DMEM solution (Dulbecco modified eagle's medium) inverted and placed at 37ºC for 24 hours to extract all leachable components. Near confluent 3T3 mouse fibroblast cells were then added to the different extracted solutions and the cell viability estimated by the standard MTT test (Mossman, 1983).

Unexposed cell cultures as well as Xeno III exposed cells were also studied under an inverted light microscope to investigate possible changes.

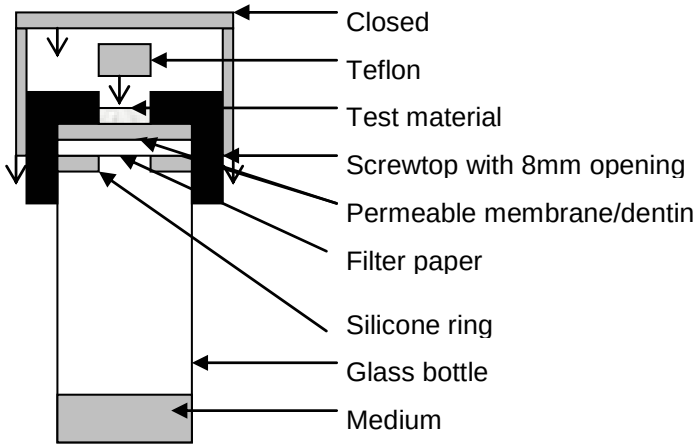

Figure 3. Schematic drawing of test bottle.

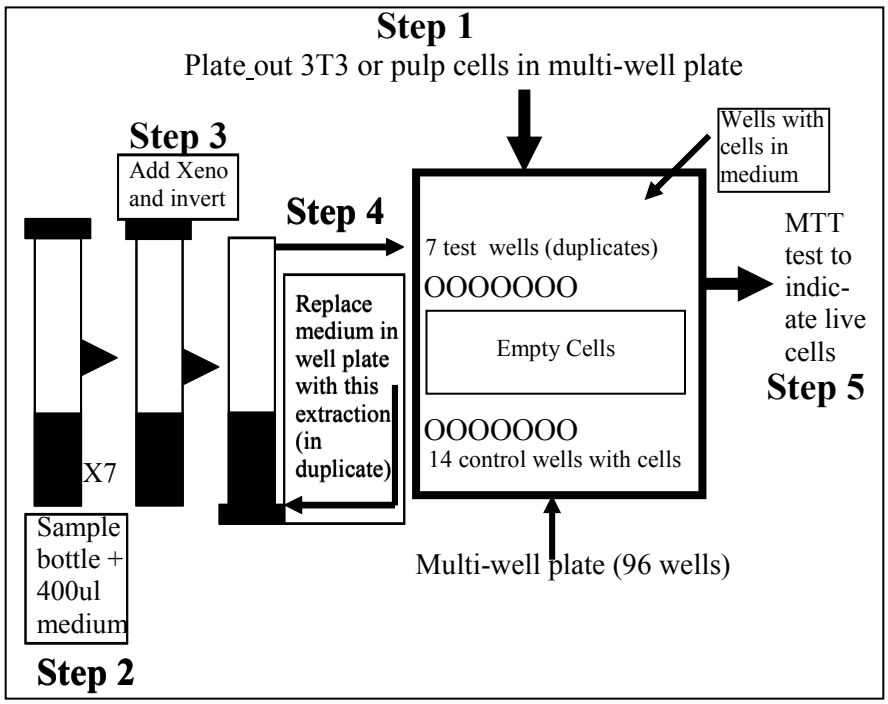

Figure 4. Schematic drawing of cytotoxicity testing of Xeno III.

RESULTS

Figure 5 shows a graph of the permeability in sec/µl against different thicknesses of dentin discs (µm). When the natural log was applied to the above graph of permeability against thickness, a straight line was obtained (Figure 6) between permeability and disc thickness.

Table 1 shows the median survival rate (and standard deviation) of 3T3 cells through dentin of different thicknesses as well as through a standard artificial membrane, when exposed to Xeno III.The Kruskal-Wallis Test was used to determine significant differences on a 5% level. No significant differences were found in the survival rate between the artificial membrane and a dentin thickness of 120 µm. However, significant differences were found between all the others.

Table 1. The median survival rates (and standard deviations) of 3T3 cells through dentin of different thicknesses as well as through a standard artificial membrane when exposed to Xeno III

Barrier Type	Artificial Membrane (n=7)	~120 µm (n=7)	~500 µm (n=7)	~750 µm (n=7)	~1000 µm (n=7)
Cell survival	25.7%	25.3%	60.7%	72.9%	100%
Standard deviation of means	0.012%	0.015%	0.32%	0.23%	0.05%

Figure 7 clearly shows differences between exposed and non-exposed balb/c mouse 3T3 fibroblast cells.

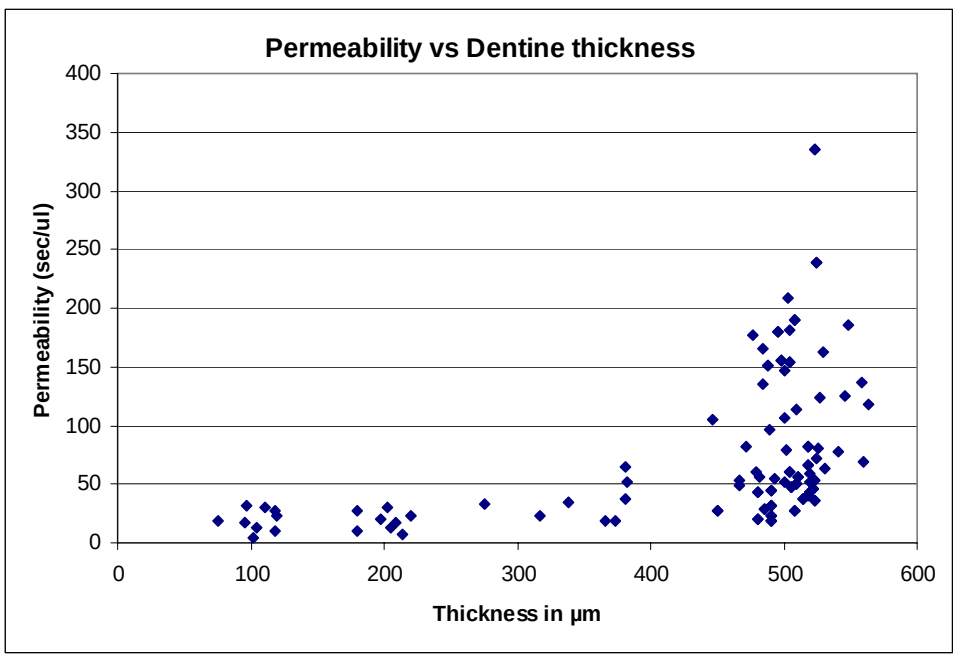

Figure 5. Graph of the permeability in sec/µl against different thicknesses of dentin discs (µm).

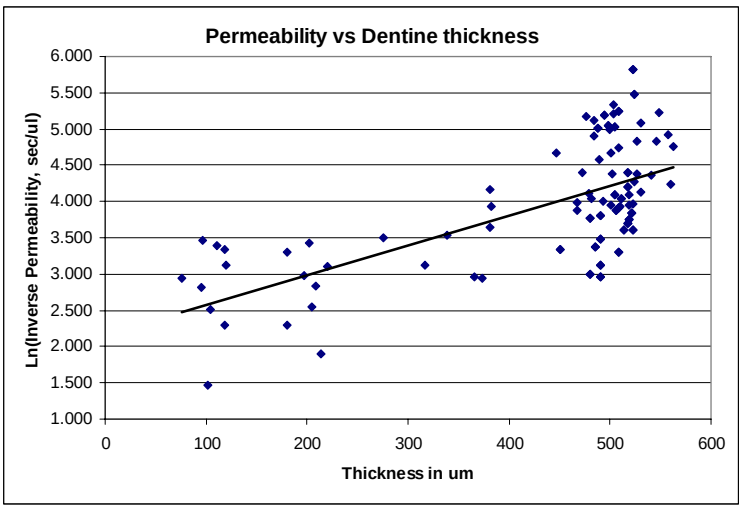

Figure 6. Natural log of permeability vs dentin thickness

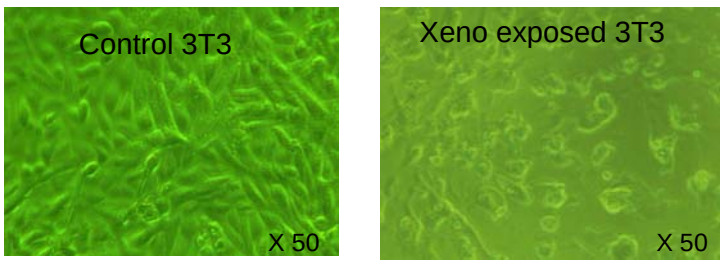

Figure 7. Typical healthy balb/c mouse 3T3 fibroblast cell (control) vs Xeno III exposed cell.

DISCUSSION

Considerable research has been done on the protective properties of dentin against toxic dental materials in the past years (Schmalz et al., 2002; Hanks et al., 1988; Hanks et al., 1994; Pashley et al., 1988; Vajrabhaya et al., 2003; Chen et al., 2003; Abou Hashieh et al., 1999). It is generally believed that dentin has the ability to protect the pulp cells against the cytotoxicity of different dental materials. This protection can be because dentin has the ability to change or alter or filter the cytotoxicity of chemical agents which are used in the dental materials.

When the relationship between discs of different thicknesses was studied (Figure 5) a large variation in the data at a specified thickness was found but still a clear link between thickness and permeability. Furthermore, it can be seen that in general the permeability of dentin discs decreased significantly at thicknesses higher than ~350 μm. However, a large spread of values can also be seen at different thicknesses with a very large spread at ~500 μm thickness group. These variations can only be attributed to individual and histological variations. Thus, it should now be born in mind that this large variation in the permeability of dentin will also result in large variations in cytotoxic tests, as no two dentin discs with the

same thickness were found with the same permeability value. However, artificial membranes could be the answer to the problem as can be seen in the following discussion.

In the cytotoxicity experiments it was found that for the artificial membrane group as well as for the dentin discs with a thickness of ~120 µm, cell viability was only around 25% compared to the control which was 100% (Table 1). As an explanation for this, it can be said that to produce the same amount of cell death in the two experiments, an equal amount of material should have leached into the imitated pulp chamber. Since the volume of applied material was kept constant, the leachable components of the material must have completely penetrated through the 120 µm discs as well as through the artificial membrane. When cytotoxicity was evaluated for the ~ 500 µm discs as barrier, cell viability was higher at 60.7%. Although this result is significantly better than for thinner discs, it still showed considerable cell death (39%). At a dentin thickness of ~750 µm the cell viability further improved to a 72.9% survival rate, while at ~1000 µm thickness the cell viability was 100% which indicates that the cytotoxic effect of Xeno was now completely illiminated at this dentin thickness. In contrast to the above finding, it was reported (Hanks et al., 1994; Murray et al., 2000; Vajrabhaya 2003) that if the remaining dentin thickness between test material and cells exceeded 500 µm, the dentin would protect the cells sufficiently by reducing the concentration of toxious materials reaching the cells thereby preventing cell death. This contrast might be due to the recent development in the chemical composition of the new generations of bonding agents which are developed for better and deeper penetration into dentin as was shown in Confocal Laser Scanning Images (Grobler et al., 2000).

In general, the standard deviations (Table 1) found for the cell viability tests also reflected the variation in the permeability of the dentin discs at various thicknesses. The lowest standard deviation was found for the artificial membrane and a dentin thickness of ~ 120 µm, which may be considered as an indication that there was no protection from these two barriers, although a variation between different dentin discs existed when it came to the shielding effect. At ~1000 µm thickness the low standard deviation can be argued as an indication of complete protection against the cytotoxicity of Xeno III which was true for all the dentin discs at this thickness.

When the cell cultures were studied under an inverted light microscope at 50x magnification, it could be seen that massive cell death had taken place in the 120-350µm groups (Figure 7). Rupture of cell membranes had taken place, causing leaking out of cytoplasm and accumulation of cell organelles around the nucleus. The nuclei of the cells were visible in the remains of the dead and rolled up fibroblasts. These stood in strong contrast to the cells at near confluence in the control which were classic spindle shaped fibroblasts with intact cell membranes and nuclei (Figure 7).

From these findings and the discussion above, it appears that for standardization purposes in *in vitro* studies, human dentin is not ideal as large variation in cell viability results can occur due to variations in dentin permeability. Although dentin discs have the advantage of directing towards the clinical situation (Pashley et al., 1988; Imazato et al., 2000), these high individual variations make it difficult to replicate in vitro experiments in order to compare the biocompatibility of different dental materials. Another factor which should be kept in mind is the depth of penetration of the material, where modern materials may have deeper penetration depths and thus are more likely to produce a cytotoxic effect. A shielding effect from ~500 µm dentin thickness, cannot be seen as benchmark to ensure that no pulp damage would take place. It is suggested that an artificial membrane should be chosen as a barrier for in vitro

cytotoxic experiments. Artificial membranes are standard, cheaper and set a level by which materials can be tested and compared world wide.

REFERENCES

Abou Hashieh I, Cosset A, Franquin JC, Camps J. In Vitro Cytotoxicity of one-step dentin bonding systems, *J Endodontics* 1999;25:89-92.

Cao, Z; Huang, L; Jiang, J. A scanning electron microscopic observation of inner carious dentin after cleansing and of the dentin-resin interface. *Quintessence Int* 1992;23:439-444.

Chen, R; Liu, C; Tseng, W; Jeng, J; Lin, C. Cytotoxicity of three dentin bonding agents on human dental pulp cells, *J Dent* 2003;31:223-229.

Geurtsen, W; Spahl, W; Müller, K; Leyhausen, G. Aqueous extracts from Dentin Adhesives containing cytotoxic chemicals. *J Biomed Mater Res(Appl Biomater)* 1999;48:772-777.

Grobler, SR; Basson, NJ; Rossouw, RJ. Shear bond strength, microleakage and anti-microbial properties of dentin bonding agent. *Am J Dent* 1996;9:120-124.

Grobler, SR; Oberholzer, TG; Rossouw, RJ. Shear bond strength, microleakage and confocal studies of four amalgam alloy bonding agents. *Quintessence Int, 2000;**31:**501-508.*

Grobler, SR; Olivier, A; Moodley, DS; vW. Kotze, TJ. Cytotoxicity of recent dentin bonding agents on mouse fibroblast cells. *Quintessence Int* 2008;39:511-516.

Hanks, CT; Graig, RG; Diehl, ML; Pashley, DH. Cytotoxicity of dental composites and other materials in a new in vitro device. *J Oral Pathol* 1988;17:396-403.

Hanks, CT; Wataha, JC; Parsell, RR; Straw, SE. Permeability of biological and synthetic molecules through dentin. *J of Oral Rehab* 1994;21:475-478.

Imazato, S; Tarumi, H; Ebi, N; Ebisu, S. Cytotoxic effects of composite restorations employing self etching primers or experimental antibacterial primers. *J Dent 2*000;28:61-67.

Murray, PE; Lumley, PJ; Ross, HF; Smith, AJ. Tooth slice organ culture for cytotoxicity assessment of dental materials. *Biomat* 2000;21:1711-1721.

Pashley, DH; Derksen, CD; Tao L. The effects of multi-step dentin bonding system on dentin permeability. *Dent Mater* 1988;4:60-63.

Schmalz, G. Agar overlay method. *Int Endod J*.1988;21:59-66.

Schmalz, G. Characterization of an in vitro dentin barrier test using a standard toxicant. *J Endod* 1994;20:592-94.

Schmalz, G; Hiller, KA; Nunez, L J; Stoll, J; Weis, K. Permeability characteristics of bovine and human dentin under different pretreatment conditions. *J Endod* 2001;27:23-30.

Schmalz, G; Schuster, U; Koch, A; Schweikel, H. Cytotoxicity of low pH- dentin bonding agents in a dentin barrier in vitro. *J Endod.* 2002;28:188-192.

Szep, S; Kunkel, A; Ronge, K; Heideman, D. Cytotoxicity of modern Dentin Adhesives- in Vitro Testing on gingival Fibroblasts. *J Biomed Mater Res* 2002;63:53-60.

Vajrabhaya, L, Pasasuk, A, Harnirattisai, C. Cytotoxicity Evaluation of single component Dentin Bonding Agents. *Oper Dent* 2003;28:440-444.

Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* 1983;65:55-63.

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Short Commentary 2

BIOCOMPATIBILITY OF DENTAL MATERIALS: ASSESSMENT OF GENETIC DAMAGE AND CELLULAR DEATH

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ABSTRACT

Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application. Taking into account the tests available in general field, genotoxicity and cytotoxicity assays are of special concern since genetic damage and cellular death play important role in carcinogenesis. The purpose of this chapter is to provide an overview on the ability of compounds currently used in dentistry such as fluoride, chlorhexidine, endodontic compounds, dental bleaching agents, glass ionomer cements, fixed orthodontic appliances, dental implants and titanium miniplates in inducing genetic damage and/or cell death based on our recent research. Some of these compounds appear capable of exerting noxious activity on the cellular machinery. The action mechanisms are discussed. Therefore, this is an area that warrants investigation since the estimation of risk of these substances with respect to genotoxicity and/or cytotoxicity certainly will improve oral health as well as will prevent oral carcinogenesis.

Keywords: genotoxicity, cytotoxicity, fluoride, chlorhexidine, endodontic compounds, dental bleaching agents, glass ionomer cements

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INTRODUCTION

Toxicology and risk assessments rely upon various testing systems to estimate risks of adverse effects from chemical exposures. Risk managers use those estimations to help protect people from potential harm (Lamb and Brown, 2000).

Nowadays, genotoxicity and cytotoxicity tests have become a fundamental tool for regulatory decisions, since the data have typically been important in regulatory schemes and decisions involving the identification of potentially DNA-damaging agents as well as those chemicals able to induce cellular death in so far as supporting concerns for carcinogenicity (USEPA, 2007). The justification for using genetic toxicology tests to predict carcinogenicity is based on the early DNA damage theory of carcinogenesis because it is irrefutable that genetic changes are essential for neoplastic transformation (Ribeiro et al. 2004b). Regarding cytotoxicity, it has been demmonstrated that cellular death induces continuous cellular proliferation trigerring non-genotoxic mechanisms of carcinogenesis as well. Therefore, the use of these assays is crucial for evaluation of potential human toxicity so that hazards can be prevented. Moreover, these data contribute to significant understanding of the mode of toxic action.

Numerous guidelines have been established worldwide such as the United States, Canada, Europe, United Kindgon, Australian, Japan and the Nortic countries. The major ones are regulatory agencies of the United States Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) that interpret the available toxicity data in risk assessments for regulatory decision-making. The basic requirements of the major guidelines are similar, but there are variations. These are mostly attributed to the scientific exposertise and mission of the agency. In recent years, efforts have been made to harmonize these guidelines, which resulted in the adoption of two major guidelines, presumably applicable worldwide. They are the International Conference on Harmonization (ICH) guidelines (ICH, 1990;1996) and the Organization for Economic Cooperation and Development (OECD) (OECD, 1997). These ICH guidelines are for pharmaceuticals (Muller et al., 1999). The OECD guidelines were co-developed with USEPA, mostly for environmental and agricultural chemicals. Many assays are able to measure genotoxicity, such as the bacterial reverse gene mutation assay (Salmonella reversion assay or Ames test), the mouse lymphoma gene mutation assay, the micronucleus test, the chromosome aberration test, and the single cell gel (comet) assay. Both guidelines (ICH and OECD) require the *Samonella* bacterial mutagenicity assay, an in vitro chromosome aberration test and an in vivo cytogenetic asssay. If a contracditory or positive response is observed in the above assays, however, additional in vitro, but often in vivo, tests would be required to assess genotoxicity further. With respect to cytotoxicity assays, there are in vitro studies, such as trypan blue exclusion assay, MTT assay or in vivo studies using rodents as well.

In light of these considerations, the purpose of the present chapter is to provide an overview on the genotoxic and/or cytotoxic potential of some most important compounds currently used in dental practice based on our recent research, i.e. fluoride, chlorhexidine, endodontic compounds, dental bleaching agents, glass ionomer cements, fixed orthodontic appliances, dental implants and titanium miniplates.

FLUORIDE

Fluoride intake in low concentrations during tooth development results in formation of dental enamel, which is more resistant to caries (Bartlett et al., 2005). According to the Center for Diseases Control, fluoridation by drinking water to prevent dental caries was considered to be one of the ten most important public health achievements of the twentieth century (Everett et al., 2002). It was established that a concentration of 0.7 ppm fluoride in drinking water reduces caries by 40–49% in primary teeth and 50-59% in permanent teeth, with no clinical appearance of adverse effects (WHO, 1984).

In last decades, some human populations are exposed to high doses of fluoride, mainly in developing countries. Herein, some studies focusing on possible genotoxic effect of excess fluoride are contradictory and inconclusive. According to some authors, lack of toxicogenetic effects was verified in Salmonella under high doses of sodium fluoride (Martin et al., 1979). Furthermore, fluoride did not induce DNA damage in mammalian cells (Matsuda, 1980; Li et al., 1989; 1995; Sato et al., 1989; Slamenova et al., 1992; Kleinsasser et al., 2001; Ribeiro et al., 2004a; 2006a). On the other hand, some authors have reported the mutagenic potential of fluoride in mice (Mohamed and Chandler, 1977). Chromosome aberrations were also increased by fluoride at doses of 25–100 μg/mL in CHO cells, in cultured human lymphocytes and fibroblasts at doses of 20–40 μg/mL and by using the short-term experimental test system in vivo (Albanesi, 1987; Aarderma et al., 1989; Velazquez-Guadarrama et al., 2005). Sodium fluoride was able to induce morphological and neoplastic transformation of Syrian hamster embryo cells (Jones et al., 1988). Yet investigators have mentioned both synergistic and antagonist effects with known mutagens (Voegel, 1973). Taking into account the few in vivo genotoxicity data available, we have designed a study aiming to evaluate a possible genotoxic potential of sodium fluoride in Wistar rats. The single cell gel (comet) assay was performed in peripheral blood leukocytes, oral mucosa and brain cells of animals treated with two different doses of sodium fluoride in drinking water (7 and 100 ppm) for six weeks. No measurable genotoxicity was found by sodium fluoride in all the cellular types evaluated (Ribeiro et al., 2004b).

Fluoride is rapidly and extensively absorbed from the gastrointestinal tract after ingestion. Owing to the action of clearance mechanisms, fluoride may be transported to the liver, kidney and urinary bladder until it is ultimately excreted. In this regard, Buzalaf et al. (2006) have recently investigated the genotoxic effects of sodium fluoride in liver, kidney and urinary bladder of Wistar rats treated with 5, 15 and 100 ppm of sodium fluoride in drinking water for six weeks. The results showed no detectable genotoxicity as depicted by the single cell gel (comet) assay. In addition, Leite et al. (2007) have showed the lack of genotoxic effect in peripheral blood, liver, kidney, urinary bladder and thyroid cells when acute fluoride at high doses was administrated to Wistar rats. In a recent study, we were also able to verify if fluoride is also able to interact with known genotoxins present in the environment such as hydrogen peroxide and alkylating agents. Such findings showed clear concentration-related increases for DNA damage in CHO cells induced by methilmetasulphonate or hydrogen peroxide. Nevertheless, at all doses tested DNA damage was not altered by previous treatment with fluoride (Ribeiro et al. 2007a). Conversely, other authors have reported DNA damage in oral mucosa cells and hepatocytes of rats exposed to NaF at 150 mg/L when compared to control group (He et al., 2006). In a recent study conducted by Zhang et al.

(2008), fluoride could induce S-phase cell-cycle arrest, up-regulation of NF-B and DNA damage in primary rat hippocampal neurons. By comparison, some authors have revealed that fluoride is able to induce necrosis and apoptosis on rat thymic cells in vitro (Matsui et al., 2007).

Considering the hallmark of apoptosis, DNA fragmentation, some authors have verified that fluoride produced large DNA fragments, possibly due to activation of caspases and p53 with consequent up-regulation of MDM2 and p21 genes and those included in the bcl-2 gene family (Anuradha et al., 2000; Morgan et al., 2002; Wang et al., 2004; Otsuki et al., 2005; Satoh et al., 2005; Lee et al., 2008). Since these genes play an important role in the apoptotic pathways, the dysregulation of apoptosis in cells induced by fluoride could be one of the mechanisms involved in the transformation from benign to malignant phenotype. Such mechanism is well documented by our research group during oral carcinogenesis (Ribeiro et al. 2005e; 2007c). In addition, studies have shown that fluoride is able to inhibit both protein and DNA synthesis in cultured mammalian cells and promotes oxidative stress and/or lipid peroxidation *in vitro* and *in vivo* (Guan et al., 2000; Anuradha et al., 2001; Ghlosh et al., 2002; Kubota et al., 2005; He et al., 2006; Zhang et al., 2007). Particularly, the inhibition of DNA synthesis may be a secondary effect of the inhibition of protein synthesis, or a result of the direct inhibition of DNA polymerase or other DNA-synthesis-associated enzymes.

CHLORHEXIDINE

Chlorhexidine is a symmetrical cationic molecule containing two 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain. In 1970, chlorhexidine was first reported as an effective antimicrobial agent by Loe and Schiott (1970). Its actions include anti-dental plaque formation, gingivitis control and root-canal disinfection. However, data about the genotoxic potential of chlorhexidine are very limited and controversial. Increased DNA damage in peripheral leukocytes and buccal cells was detected by the single cell gel (comet) assay in individuals that rinsed their mouths with chlorhexidine at 0.12% concentration (Eren et al., 2001). Gene mutations were also induced by the breakdown products of chlorhexidine in microorganisms (Prasad, 1970). Furthermore, the mouse lymphoma assay detected a positive response after chrlohexidine exposure *in vitro* (Withrow et al., 1989; Mitchell et al., 1997). Nevertheless, negative results were detected in the SOS chromotest and UMU test (Sakagami et al., 1988; Klimm et al., 1989). An earlier study using SHE cells treated with chlorhexidine at concentrations up to 10 μM also showed no increase of sister-chromatid exchanges (Miyachi and Tsutsui, 2005).

On the basis of inappropriate evidence about the genotoxic potential of chrorhexidine, we designed an experimental study in order to evaluate whether this antiseptic compound can cause DNA damage in rat oral mucosa and peripheral blood cells in vivo (Ribeiro et al., 2004c). Statistically significant increase of DNA damage was detected either in peripheral leukocytes or in oral mucosa cells of the animals exposed to chlorhexidine at 0.12% concentration (Ribeiro et al., 2004c). However, it is not still clear how chlorhexidine is absorbed from the oral mucosa and gastrointestinal tract and then promotes these biological actions. In an attempt to elucidate this issue, the micronucleus test was applied in this setting. The results showed no increase of micronucleated cells in reticulocytes, indicating that

chlorhexidine did not induce chromosome breakage or loss in erythrocytes. Later, we assayed the genotoxic potential of chlorhexidine at concentrations ranging from 0.01 to 1 % on CHO cells. It was clearly observed that chlorhexidine at all concentrations tested did not cause an increase of DNA injury (Ribeiro et al., 2005a). However, it is important to emphasize that in vitro test systems do not consider the complex homeostatic situation that occurs in vivo. In this sense, we were able to evaluate if chlorhexidine is able to induce genetic damage in multiple organs, such as liver, kidney and urinary bladder. Again, this compound caused genetic damage in kidney cells (Grassi et al., 2007). In a recent study conducted by Gianelli et al. (2008), chlorhexidine was highly cytotoxic in vitro and invite to a more cautioned use of the antiseptic in the oral surgical procedures. This was confirmed by others (Hidalgo and Domingues, 2001; De Sousa et al., 2007).

Some authors have provided evidence on the mode of action of chlorhexidine. The interaction between chlorhexidine and metals is assumed (Warner et al., 1993). According Negrello-Newton et al. (2004), the possibility of an interaction between chlorhexidine and iron must be considered. Iron mobilization has long been known to induce oxidative stress, leading to lipid peroxidation, oxidative DNA damage, and possibly plays a role in iron-induced carcinogenesis (Rocha et al., 2000). Taken as a whole, we suggested that chlorhexidine is an agent able to exert genotoxic effects on mammalian cells. It is important to stress that people are not able to absorb chlorhexidine in the form of mouthwash (the main presentation form used in dentistry), because they tend to spit it out, as well as this compound does not accumulate in body. Therefore, DNA breakage induced by chlorhexidine should be regarded as transient. However, continous exposure may be more predictive of genetic toxicity as far as to offer some risk for humans.

ENDODONTIC COMPOUNDS

The main purposes of endodontic therapy are to eliminate microorganisms in root canals and fillings in order to restore dental function. To best of our knowledge, only a few studies on putative genotoxicity of endodontic agents have been conducted and those report with divergent findings. Huang et al. (2001) have argued that zinc oxide eugenol-based sealers do not induce DNA damage in oral carcinoma cells in vitro. However, they have detected a dose-dependent increase of genotoxicity when cells were exposed to resin-based sealers. These data are largely of the same magnitude as those reviewed by Geurtsen et al. (1997). Later, Tai et al. (2002) found that N2-, AH26-, and AH-Plus-based root canal sealers exhibited genotoxicity by causing DNA single-strand breaks. The same findings were encounted by Schweikl et al. (1998a) by means of AMES test. However, Huang et al. (2002a,b) have demmonstrated that resin-based sealers such as AH26 and AH-Plus sealers exhibited cytotoxicity and a dose-dependent increase of DNA damage in vitro assessed by single cell gel (comet) assay. More recently, this research group has evidenced no aparent genomic damage induced by root canal sealers (Huang et al., 2004).

In human dental pulp fibroblasts exposed to phenolic compounds, potent antimicrobial agents, no DNA breakage was detected (Chang et al., 2000; do Céu Silva et al., 2003). Conversely, these same substances were able to induce unscheduled DNA synthesis in a concentration-dependent manner on SHE cells (Hamaguchi and Tsutsui, 2000). Recently, we

investigated whether formocresol, paramonochlorophenol or calcium hydroxide could induce DNA damage in mouse lymphoma cells and primary human fibroblasts by the single cell gel (comet) assay in vitro. These materials are widely used in endodontic practice to eradicate bacteria and consequently to produce root canal disinfection. Our data demonstrated that the single treatment with formocresol, paramonochlorofenol and calcium hydroxide at concentrations of 20, 40 and 80 µg/mL did not induce strand breaks in DNA (Ribeiro et al., 2004d). Similar results were observed in CHO cells after exposure to formocresol, paramonochlorophenol and calcium hydroxide adjusted to 100 µg/mL (Ribeiro et al., 2005a). In fact, it has been reported that chlorophenol failed to promote unscheduled DNA synthesis in the presence or absence of exogenous metabolic activation (Hagiwara et al., 2006), although cresol induced unscheduled DNA synthesis in the presence of exogenous metabolic activation (Hamaguchi and Tsutsui, 2000). By contrast, statistically significant differences in the frequencies of sister-chromatid exchanges were observed in SHE cells treated with chlorophenol or formaldehyde (Miyachi and Tsutsui, 2005) and chromossomal aberrations were induced by formocresol, with chlorophenol being a significant chromosomal aberration inducer in the presence of exogenous metabolic activation only (Hagiwara et al., 2006). Besides cresol, formocresol is also composed of formaldehyde that is a known genotoxic and cytotoxic substance (Casanova et al., 1994; Lovschall et al., 2002). The primary genotoxic effect seems to be the formation of DNA-protein crosslinks (Merck and Speit, 1999). In a recent study designed by Ramos et al. (2008), formocresol induced DNA-protein cross-link and an increased frequency of micronucleus in bone marrow cells in vitro.

We were able to evaluate whether some gutta-percha solvents, such as chloroform and eucalyptol, are able to cause genetic damage in vitro. Our results demmonstrated that both compounds do not promote DNA breakage in CHO or mouse lymphoma cells after single exposure (Ribeiro et al. 2006h; 2007b). Taken together, it seems that some endodontic compounds induce genetic damage as depicted by genotoxicity results. Therefore, the use of these compounds should be cautious in dentistry.

To ensure normal growth control and integrity in DNA molecule, cells have developed many strategies to manage stress (Martinez et al. 2003). However, a failure on some of these defense mechanisms may lead to the development of degenerative diseases such as cancer. Nowadays, it is well established that exogenous agents can modify the cellular DNA, along with other cellular components (Martinez et al. 2003). In this way, it would be useful to know whether, and to what extent, these antimicrobial endodontic compounds possess synergistic and/or antagonist effects with known genotoxins present in our environment. Thus, we have performed assays to investigate the ability of formocresol, paramonochlorophenol and calcium hydroxide to modulate the genotoxic effects induced by the oxidatively damaging agent hydrogen peroxide and the alkylating agent methyl methanesulfonate in vitro. The addition of methyl methanesulfonate in increasing concentrations to cells incubated with formocresol, paramonochlorophenol or calcium hydroxide did not contribute to genetic damage (Ribeiro et al., 2006f). Similar findings were observed with respect to hydrogen peroxide for all endodontic compounds evaluated (Ribeiro et al., 2006f).

In the 1990s, mineral trioxide aggregate was developed as a root-end-filling material. A number of biocompatibility studies has been conducted either in vitro or in vivo showing that mineral trioxide aggregate has good sealing ability and tissue healing properties. Nevertheless, genotoxicity data are needed to complete risk assessment of mineral trioxide aggregate. Herein, we have investigated whether regular and white mineral trioxide aggregate

can induce DNA damage *in vitro* by means of mouse lymphoma and CHO cells. The results showed that the materials tested did not induce DNA strand breaks at concentrations ranging from 1–1000 μg/mL as depicted by the single cell gel (comet) assay (Ribeiro et al., 2005b; 2006b,c). These findings confirmed and extended the data already published showing a good biocompatibility of mineral trioxide aggregate.

Nowadays, studies have compared mineral trioxide aggregate with Portland cement. The findings suggest that they seem almost identical macroscopically, microscopically and by x-ray diffraction analysis, and contain the same chemical elements. This information indicates that Portland cement has the potential to be used as a less expensive root-end-filling material in dental practice. We have observed the absence of genotoxicity in rodent neoplastic cells exposed to regular and white Portland cements at concentrations ranging from 1–1000 μg/mL To verify the genotoxicity in ordinary human cells, we exposed lymphocytes to these compounds in vitro (Ribeiro et al., 2006c). Mineral trioxide aggregate at concentrations up to 1000μg/mL did not induce genotoxic effect (Braz et al., 2006). Furthermore, no DNA damage was found for ordinary and white Portland cements (Braz et al., 2006). In summary, these data are consistent with the notion that mineral trioxide aggregate and Portland cements had no genotoxic effect.

DENTAL BLEACHING AGENTS

Dental bleaching is a simple and conservative procedure for esthetic restoration of vital or non-vital discolored teeth. There are many bleaching agents commercially available with various constituents, such as hydrogen peroxide and carbamide peroxide. Carbamide peroxide decomposes to produce hydrogen peroxide, which may be considered the active ingredient of choice for bleaching because of its low molecular weight and its ability to denature proteins. Nevertheless, a number of studies have demonstrated the risk of tissue damage from contact of these agents with the oral mucosa (Floyd, 1997). In fact, hydrogen peroxide is able to interact both directly with DNA and through highly reactive oxygen and radical species causing extensive oxidative DNA damage (Daroui et al. 2004). So far, oxidative DNA damage has been recognized as a major cause of cell death and mutations in all aerobic organisms. In humans, oxidative DNA damage is also considered an important promoter of cancer (Bjelland and Seeberg, 2003). Conversely, few efforts have been made to establish the putative genotoxicity of dental bleaching agents. Zouain-Ferreira et al. (2002) have evaluated the genotoxic effects of four commercial dental bleaching agents: Insta-Brite, Karisma, Opalescence and Whiteness using bacterial mutation assay as end-point. The bleaching agents were capable of inducing DNA damage. The authors concluded that dental bleaching compounds can generate biological effects like those from ionizing radiations. To verify the genotoxicity of these agents on eukaryotic cells, experiments were designed by our group. The results showed that all dental bleaching tested (Clarigel Gold; Whitespeed; Nite White Excel 2; Magic Bleaching; Whiteness HP and Lase Peroxide) induced DNA damage as detected by the single cell gel (comet) assay either to CHO cells or to L5178Y mouse lymphoma cells (Ribeiro et al., 2005d; 2006d). A clear dose-response relationship was observed, with the strongest effect obtained at the highest dose of hydrogen peroxide (Whiteness HP and Lase Peroxide, at 35% concentration). In fact, the hydrogen peroxide

molecule easily permeates the cell membrane and is transformed into hydroxyl radicals by a non-enzymatic process in the presence of metal ions (Fe2+ or Cu2+) occurring in the cytoplasm, known as the Haber-Weiss or Fenton reaction. Hydroxyl radicals, a potent derived free radical species, can induce single-strand breaks, double-strand breaks, alkali-labile sites and various species of oxidized purines and pyrimidines. Other reactive oxygen species derived from hydrogen peroxide can also interact with DNA and induce a broad spectrum of DNA lesions. Furthermore, the cytotoxic evaluation was made using dental bleachings. Such findings pointed out that dental bleachings are cytotoxic as well as carbamide peroxide products (Woolverton et al., 1993; Koulaouzidou et al., 1998). Based on these results presented, therefore, it is timely to postulate that dental blechings exert cytotoxic and genotoxic effects on mammalian cells. Hence, dental bleachings should be used only when necessary in dental practice since these compounds cause noxious activies as a result of extensive cellular damage.

GLASS IONOMER CEMENTS

Since glass ionomer cements were first introduced in the early 70s by Wilson and Kent (1972), they have been used extensively in dentistry as restorative materials and adhesives for composite restorations. Their usage also includes prosthetic and orthodontic devices. Some studies have demonstrated that glass ionomer cements are able to induce DNA damage in various test systems such as the bacterial UMU-test, eukaryotic DNA synthesis inhibition test, in vivo alkaline filter elution technique as well as sister chromatid exchange test in human lymphocytes (Heil et al., 1996; Stea et al., 1998; Muller et al., 2003). However, negative results were also observed under in vitro experiments (Schweikl et al., 1994; 1998b). Such discrepancies have been attracting attention to new studies on genotoxicity of these cements. Data obtained in our laboratory using CHO cells demonstrated that the powder from Ketac Molar displayed genotoxicity only at the maximum concentration tested (100 μg/mL) (Ribeiro et al., 2006e). Similarly, liquid from Vitrebond at 0.1% dilution caused an increase of DNA damage (Ribeiro et al. 2006g). These data are in accordance with those described by Kleinsasser et al. (2004; 2006) who found only a small genotoxicity induced by 2-hydroxyethyl methacrylate (HEMA), a component of liquid Vitrebond. No significant DNA damage was noted at concentrations possibly relevant to in vivo situations (10-4 M) (Spahl et al., 1998) Nevertheless, studies reported that HEMA induced apoptotic death, probably affecting the intrinsic apoptotic pathway as well as generating oxidative stress (Paranjpe et al., 2005; Becher et al., 2006; Lee et al., 2006; Samuelsen et al., 2007). In conclusion, these results support the rationale that some glass ionomer cements induced DNA migration detected by single cell gel (comet) assay as a sign for limited genotoxic effects in higher concentrations. High cytotoxicity was evidenced either in vitro or in vivo (De Souza Costa et al., 2003; Lan et al., 2003; Souza et al., 2006). With the highest levels of DNA migration being combined with elevated cytotoxic effects, therefore, a low in vivo noxious activity could be observed. Further studies must be conducted with isolated substances of these glass ionomer cements in order to clarify the action mechanism.

OTHER DENTAL MATERIALS

Nowadays, interest in the amount of metal ion intake in contact with oral tissues from dental alloys has grown. Fixed orthodontic appliances from brackets, bands, archwires made of stainless steel, nickel-titanium, nickel-cobalt alloys, and dental implants, or titanium miniplates can release metal ions. Faccioni et al. (2003) showed that nickel and cobalt released from fixed orthodontic appliances can induce DNA damage in oral mucosa cells in vivo. The same results were found using gengival fibroblastsin vitro (Tomakidi et al., 2000). By comparison, our data pointed out absence of genotoxicity evaluated from eluates obtained from endosseous implants in vitro (Ribeiro et al., 2007d) or even induced by titanium miniplates in vivo (Piozzi et al., 2008).

At last, we were able to evaluate whether some radiopacifiers widely used in clinical practice are able to induce genetic damage in primary human cells in vitro. Human peripheral lymphocytes obtained from healthy volunteers were exposed to barium sulphate ($BaSO_{4)}$, zirconium oxide ($ZnO_{2)}$ and bismuth oxide ($Bi_2O_{3)}$ at final concentrations ranging from 1 to 1000 μg/mL.The results pointed that all tested compounds did not induce DNA breakage in human peripheral lymphocytes (Braz et al., 2008).

CONCLUDING REMARKS

In this review, we have highlighted recent advances on the genotoxic and/or cytotoxic potential of some compounds widely used in dental practice. Although their genotoxicity and cytotoxicity as well as possible action mechanisms have been purposed to some dental materials, much remains to be examined, specially in vivo studies for those compounds that dysplayed in vitro positive response by means of medium-term carcinogenesis assays. In addition, the role of these compounds concerning the interference on cellular signal pathways, gene expression profiles, and epygenetic mechanisms is welcomed. Therefore, this is an area that warrants investigation since the estimation of risk of these substances will be added to those used for regulatory purposes in improving oral health and preventing oral carcinogenesis.

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REFERENCES

Aardema MJ, Gibson DP, LeBoeuf RA (1989). Sodium fluoride-induced chromosome aberrations in different stages of the cell cycle, a proposed mechanism. *Mutation Research* 223,191-203.

Albanese R (1987). Sodium fluoride and chromosome damage (in vitro human lymphocyte and in vivo micronucleus assays). *Mutagenesis* 2,497-499.

Anuradha CD, Kanno S, Hirano S (2000). Fluoride induces apoptosis by caspase-3 activation in human leukemia HL-60 cells. *Archives of Toxicology* 74,226-230.

Anuradha CD, Kanno S, Hirano S (2001). Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. *Free Radical Biology and Medicine* 31,367-373.

Bartlett JD, Dwyer SE, Beniash E, Skobe Z, Payne-Ferreira TL (2005). Fluorosis, a new model and new insights. *Journal of Dental Research* 84,832-836.

Becher R, Kopperud HM, Al RH, Samuelsen JT, Morisbak E, Dahlman HJ, Lilleaas EM, Dahl JE (2006). Pattern of cell death after in vitro exposure to GDMA, TEGDMA, HEMA and two compomer extracts. *Dental Materials* 22, 630-640.

Bjelland S, Seeberg E (2003). Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutation Research* 29, 37-80.

Braz MG, Camargo EA, Salvadori DM, Marques ME, Ribeiro DA (2006). Evaluation of genetic damage in human peripheral lymphocytes exposed to mineral trioxide aggregate and Portland cements. *Journal of Oral Rehabilitation* 33,234-239.

Braz MG, de Castro Marcondes JP, Matsumoto MA, Duarte MA, Salvadori DM, Ribeiro DA.Genotoxicity in primary human peripheral lymphocytes after exposure to radiopacifiers in vitro. *J Mater Sci Mater Med* 2008;19:601-5.

Buzalaf MA, Salvadori DM, Marques ME, Caroselli EE, Leite AL, Camargo EA, Ribeiro DA (2006). *Bulletin of Environmental Contamination and Toxicology* 77,700-706.

Casanova M, Morgan KT, Steinhagem WH, Everitt JI, Popp JA, Heck Hd'A. DNA-protein cross-links and cell replication at specific sites in the nose of F344 rats exposed subchronically to formaldehyde (1994). *Fundamental Apply Toxicology* 23,525-536.

Chang YC, Tai KW, Huang FM, Huang MF (2000). Cytotoxic and nongenotoxic effects of phenolic compounds in human pulp cell cultures. *Journal of Endodontics* 26,440-443.

Daroui P, Desai SD, Li TK, Liu AA, Liu LF (2004). Hydrogen peroxide induces topoisomerase I-mediated DNA damage and cell death. *Journal of Biological Chemistry* 279, 14587-94.

de Souza Costa CA, Hebling J, Garcia-Godoy F, Hanks CT (2003). In vitro cytotoxicity of five glass-ionomer cements. *Biomaterials.* 24, :3853-8.

de Souza LB, de Aquino SG, de Souza PP, Hebling J, Costa CA (2007). Cytotoxic effects of different concentrations of chlorhexidine. *American Journal of Dentistry* 20, 400-4.

do Ceu Silva M, Gaspar J, Silva ID, Leao D, Rueff J (2003). Induction of chromosomal aberrations by phenolic compounds, possible role of reactive oxygen species. *Mutation Research* 540,29-42.

Eren K,Ozmeric N, Sardas S (2001). Monitoring of buccal epithelial cells by alkaline comet assay (single cell gel electrophoresis technique) in cytogenetic evaluation of chlorhexidine. *Clinical Oral Investigation* 6,150-154.

Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, Martinez-Mier EA, Warrick JM (2002). Dental fluorosis, variability among different inbred mouse strains. *Journal of Dental Research* 81,794-798.

Faccioni F, Franceschetti P, Cerpelloni M, Fracasso ME (2003) .In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa

cells.*American Journal Orthodontics Dentofacial Orthopedics* 124, 687-93; discussion 693-4.

Floyd RA (1997). The effect of peroxides and free radicals on body tissues *JADA* 128;37S-40S.

Geurtsen W, Leyhausen G (1997). Biological aspects of root canal filling materials-histocompatibility,cytotoxicity, and mutagenicity. *Clinical Oral Investigation* 1,5-11.

Ghosh D, Das Sarkar S, Maiti R, Jana D, Das UB (2002). Testicular toxicity in sodium fluoride treated rats, association with oxidative stress. *Reprodutive Toxicology* 16,385-390.

Giannelli M, Chellini F, Margheri M, Tonelli P, Tani A (2008). Effect of chlorhexidine digluconate on different cell types: a molecular and ultrastructural investigation. *Toxicology In Vitro.* 22, 308-17.

Grassi TF, Camargo EA, Salvadori DM, Marques ME, Ribeiro (2007). DNA damage in multiple organs after exposure to chlorhexidine in multiple organs. *International Journal of Hygiene and Environmental Health* 210,163-167.

Guan ZZ, Xiao KQ, Zeng XY, Long YG, Cheng YH, Jiang SF, Wang YN (2000). Changed cellular membrane lipid composition and lipid peroxidation of kidney in rats with chronic fluorosis. *Archives of Toxicology* 74,602-608.

Hagiwara M, Watanabe E, Barrett JC, Tsutsui T (2006). Assessment of genotoxicity of 14 chemical agents used in dental practice, Ability to induce chromosome aberrations in Syrian hamster embryo cells. *Mutation Research* 603,111-120.

Hamaguchi F, Tsutsui T (2000). Assessment of genotoxicity of dental antiseptics, ability of phenol, guaiacol, p-phenolsulfonic acid, sodium hypochlorite, p-chlorophenol, m-cresol or formaldehyde to induce unscheduled DNA synthesis in cultured Syrian hamster embryo cells. *Japanese Journal of Pharmacology* 83,273-276.

He LF, Chen JG (2006). DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes. *World Journal of Gastroenterology* 12,1144-1148.

Heil J, Reifferscheid G, Waldmann P, Leyhausen G, Geurtsen W (1996). Genotoxicity of dental materials. *Mutation Research* 368,181-194.

Hidalgo E, Dominguez C (2001). Mechanisms underlying chlorhexidine-induced cytotoxicity. *Toxicology In Vitro.* 15, 271-276.

Huang FM, Tai KW, Chou MY, Chang YC (2002). Cytotoxicity of resin-, zinc oxide-eugenol-, and calcium hydroxide-based root canal sealers on human periodontal ligament cells and permanent V79 cells. *International Endodontic Journal* 35,153-158b.

Huang TH, Ding SJ, Hsu TZ, Lee ZD, Kao CT (2004). Root canal sealers induce cytotoxicity and necrosis. *Journal of Materials Science Materials in Medicine* 15,767-771.

Huang TH, Lee H, Kao CT (2001). Evaluation of the genotoxicity of zinc oxide eugenol-based, calcium hydroxide-based, and epoxy resin-based root canal sealers by comet assay. *Journal of Endodontics*;27,744-748.

Huang TH, Yang JJ, Li H, Kao CT (2002). The biocompatibility evaluation of epoxy resin-based root canal sealers in vitro. *Biomaterials* 23,77-83a.

ICH. International Conference on Harmonization (1996). Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals*. Federal Registration* 61, 18198-18202.

ICH. International Conference on Harmonization (1997). Guidance on genotoxicity: a standard battery for genotoxicity testing for pharmaceuticals. *Federal Registration* 62, 62472-62475.

Jones, CA, Callaham MF, Huberman E (1988). Sodium fluoride promotes morphological transformation of Syrian hamster cells. *Carcinogenesis* 9,2279-84.

Kleinsasser NH, Schmid K, Sassen AW, Harreus UA, Staudenmaier R, Folwaczny M, Glas J, Reichl FX (2006). Cytotoxic and genotoxic effects of resin monomers in human salivary gland tissue and lymphocytes as assessed by the single cell microgel electrophoresis (Comet) assay. *Biomaterials* 27,1762-1770.

Kleinsasser NH, Wallner BC, Harreus UA, Kleinjung T, Folwaczny M, Hickel R, Kehe K, Reichl FX (2004). Genotoxicity and cytotoxicity of dental materials in human lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. *Journal of Dentistry* 32,229-234.

Kleinsasser NH, Weissacher H, Wallner BC, Kastenbauer ER, Harreus UA (2001). Cytotoxicity and genotoxicity of fluorides in human mucosa and lymphocytes. *Laryngorhinootologie* 80,187-190.

Klimm W, Janz S, Gabert A (1989). *Experimental research on the genotoxicity of various root canals antiseptics in the SOS chromotest.* Dtsch Zahn Mund Kieferheilkd Zentralbl 77,27-30.

Koulaouzidou E, Lambrianidis T, Konstantinidis A, Kortsaris AH (1998). In vitro evaluation of the cytotoxicity of a bleaching agent. *Endodontic Dental Traumatology* 14, 21-5.

Kubota K, Lee DH, Tsuchiya M, Young CS, Everett ET, Martinez-Mier EA, Snead ML, Nguyen L, Urano F, Bartlett JD (2005). Fluoride induces endoplasmic reticulum stress in ameloblasts responsible for dental enamel formation. *Journal of Biology and Chemistry* 280,23194-2202.

Kuehl DW, Serrano S, Naumann S (1994). Identification of potentially mutagenic contaminants in the aquatic environmental by liquid chromatographic-thermospray mass spectrometric characterization of in vitro DNA adducts. *Journal of Chromatography A* 684, 1113-9.

Lamb JC, Brown SM (2000). Chemical testing strategies for predicting health hazards to children. *Reprodutive Toxicology* 14,83-94.

Lan WH, Lan WC, Wang TM, Lee YL, Tseng WY, Lin CP, Jeng JH, Chang MC (2003). Cytotoxicity of conventional and modified glass ionomer cements. *Operative Dentistry* 28, 251-9.

Lee DH, Lim BS, Lee YK, Ahn SJ, Yang HC (2006). Involvement of oxidative stress in mutagenicity and apoptosis caused by dental resin monomers in cell cultures. *Dental Materials* 22, 1086-1092.

Lee JH, Jung JY, Jeong YJ, Park JH, Yang KH, Choi NK, Kim SH, Kim WJ (2008) Involvement of both mitochondrial and death receptor dependent apoptotic pathways regulatedby bcl-2 family in sodium fluoride induced apoptosis of the human gingival fibroblasts *Toxicology* 243: 340-347.

Leite AL, Santiago Jr JF, Levy FM, Maria AG, Fernandes MS, Salvadori DM, Ribeiro DA, Buzalaf MA (2007). Absence of DNA damage in multiple organs (blood, liver, kidney, urinary bladder and thyroid) after acute fluoride exposure in rats. *Human and Experimental Toxicology*, in press.

Li Y, Liang CK, Katz BP, Brizendine EJ, Stookey GK (1995). Long-term exposure to fluoride in drinking water and sister chromatid exchange frequency in human blood lymphocytes. *Journal of Dental Research* 74,1468-1474.

Li YM, Zhang W, Noblitt TW, Dunipace AJ, Stookey GK (1989). Genotoxic evaluation of chronic fluoride exposure, sister-chromatid exchange study. *Mutation Research* 227,159-165.

Loe H, Schiott CR (1970). The effect of mouthrinses and topical application of chlorhexidine on of dental plaque and gingivitis in man. *Journal of Periodontal Research* 5,79-83.

Lovschall H, Eiskjaer M, Arenholt-Bindslev D (2002). Formaldehyde cytotoxicity in three human cell types assessed in three different assays. *Toxicology In Vitro.* 16, 63-9.

Martin GR, Brown KS, Matheson DW, Lebowitz H, Singer L, Ophaug R (1979). Lack of cytogenetic effects in mice or mutations in Salmonella receiving sodium fluoride. *Mutation Research* 66,159-167.

Martinez GR, Loureiro AP, Marques SA, Miyamoto S, Yamaguchi LF, Onuki J, Almeida EA, Garcia CCM, Barbosa LF, Medeiros MHG, Di Mascio P (2003). Oxidative and alkylating damage in DNA. *Mutation Research* 544, 115-27.

Matsuda A (1980). *Cytogenetic effects of fluoride on human lymphocytes in vitro*. Koku Eisei Gakkai Zasshi 30,312-328.

Matsui M, Morimoto M, Horimoto K, Nishimura Y (2007) Some characteristics of fluoride induced- cell death in rat thymocytes: cytotoxicity of sodium fluoride. *Toxicol In vitro* 21:1113-1120.

Merck O, Speit G (1999). Detection of crosslinks with the comet assay in relationship to genotoxicity and cytotoxicity. *Environmental and Molecular Mutagenesis* 39,167-172.

Mitchell AD, Auletta AE, Clive D, Kirby PE, Moore MM, Myhr BC (1997). The L5178Y/rk+/- mouse lymphoma specific gene and chromosomal mutation assay. A phase III report *of the US environmental protection agency gene-tox program.* Mutation Research 394,177-303.

Miyachi T, Tsutsui T (2005). Ability of 13 chemical agents used in dental practice to induce sister-chromatid exchanges in Syrian hamster embryo cells. *Odontology* 93,24-29.

Mohamed H, Chandler ME (1977). Cytological effects of sodium fluoride on mice, presented during hearings. The National Cancer Program. Part 2. *Fluoridation of public drinking water*. Washington, 95th US Congress 21,42-60.

Morgan KT, Ni H, Brown HR, Yoon L, Qualls CW Jr, Crosby LM, Reynolds R, Gaskill B, Anderson SP, Kepler TB, Brainard T, Liv N, Easton M, Merrill C, Creech D, Sprenger D, Conner G, Johnson PR, Fox T, Sartor M, Richard E, Kuruvilla S, Casey W, Benavides G (2002). Application of cDNA microarray technology to in vitro toxicology and the selection of genes for a real-time RT-PCR-based screen for oxidative stress in Hep-G2 cells. *Toxicology Pathology* 30,435-451.

Muller BP, Eisentrager A, Jahnen-Dechent W, Hollender J (2003). Effect of sample preparation on the in vitro genotoxicity of a light curable glass ionomer cement *Biomaterials* 24,611-617.

Muller L, Kikuchi Y, Probst G, Strechtman L, Shimada H, Sofuni T, Tweats D (1999). ICH-harmonized guidelines on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. *Mutation Research* 436, 195-225.

Negrelo Newton AP, Cadena SM, Merlin Rocha ME, Skare Carnieri EG, Martinelli de Oliveira MB (2004). New data on biological effects of chlorhexidine, Fe2+ induced lipid peroxidation and mitochondrial permeability transition. *Toxicology Letters* 151,407-416.

OECD. Organization for economic co-operation and development. (1997). *OECD Guidelines for the testing of chemicals.* TG 471-486, Paris, France.

Otsuki S, Morshed SR, Chowdhury SA, Takayama F, Satoh T, Hashimoto K, Sugiyama K, Amano O, Yasui T, Yokote Y, Akahane K, Sakagami H (2005). Possible link between glycolysis and apoptosis induced by sodium fluoride. *Journal of Dental Research* 84,919-923.

Paranjpe A, Bordador LC, Wang MY, Hume WR, Jewett A (2005). Resin monomer 2-hydroxyethyl methacrylate (HEMA) is a potent inducer of apoptotic cell death in human and mouse cells. *Journal of Dental Research* 84,172-177.

Piozzi R, Ribeiro DA, Padovan LE, Filho HN, Matsumoto MA (2008). Genotoxicity and cytotoxicity in multiple organs induced by titanium miniplates in Wistar rats.*J Biomed Mater Res* A 2008 , in press.

Prasad I (1970). Mutagenic effects of the herbicide 3', 4' dichloropropionanilide and its degradation products. *Canadian Journal of Microbiology* 16,369-72.

Ramos ME, Cavalcanti BC, Lotufo LV, Moraes MO, Cerqueira ME, Pessoa C (2008) Evaluation of mutagenic effects of formocresol: detection of DNA-protein cross links and micronucleus in mouse bone marrow. Oral Surgery Oral Medicine Oral Pathology Oral *Radiology Endodontology* 105:398-404.

Ribeiro DA, Alves de Lima PL, Marques MEA, Salvadori DMF (2006a). Lack of DNA damage induced by fluoride on mouse lymphoma and human fibroblast cells by single cell gel (comet) assay. *Brazilian Dental Journal* 2,27-31.

Ribeiro DA, Bazo AP, da Silva Franchi CA, Marques ME, Salvadori DM (2004c). Chlorhexidine induces DNA damage in rat peripheral leukocytes and oral mucosal cells. *Journal of Periodontal Research* 39,358-361.

Ribeiro DA, Duarte MA, Matsumoto MA, Marques ME, Salvadori DM (2005b). Biocompatibility in vitro tests of mineral trioxide aggregate and regular and white Portland cements. *Journal of Endodontics* 31,605-607.

Ribeiro DA, Kitakawa D, Domingues MA, Cabral LA, Marques ME, Salvadori DM. (2007c). Survivin and inducible nitric oxide synthase production during 4NQO-induced rat tongue carcinogenesis: A possible relationship. *Experimental and Molecular Pathology,* in press.

Ribeiro DA, Marques ME, de Assis GF, Anzai A, Poleti ML, Salvadori DM (2004b). No relationship between subchronic fluoride intake and DNA damage in Wistar rats. *Caries Research* 38,576-579.

Ribeiro DA, Marques ME, Salvadori (2006h). In vitro cytotoxic and non-genotoxic effects of gutta percha solvents by single cell gel (comet) assay. *Brazilian Dental Journal* 17, 228-232.

Ribeiro DA, Marques ME, Salvadori DM (2004d). Lack of genotoxicity of formocresol, paramonochlorophenol, and calcium hydroxide on mammalian cells by comet assay. *Journal of Endodontics* 30,593-536.

Ribeiro DA, Marques ME, Salvadori DM (2005c). Assessment of genetic damage induced by dental bleaching agents on mouse lymphoma cells by single cell gel (comet) assay. *Journal of Oral Rehabilitation* 32,766-771.

Ribeiro DA, Marques ME, Salvadori DM (2006f). Antimicrobial endodontic compounds do not modulate alkylation-induced genotoxicity and oxidative stress in vitro. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* 102,32-36.

Ribeiro DA, Marques ME, Salvadori DM (2006g). Biocompatibility of glass ionomer cements using mouse lymphoma cells in vitro. *Journal of Oral Rehabilitation*, 33,912-917.

Ribeiro DA, Marques ME, Salvadori DM (2007a). Lack of effect of prior treatment with fluoride on genotoxicity of two chemical agents in vitro. *Caries Research* 41, 239-243.

Ribeiro DA, Marques MEA, Salvadori DMF (2006d). *Study of DNA damage induced by dental bleaching agents in vitro*. Pesquisa Odontologica Brasileira 20,47-51.

Ribeiro DA, Matsumoto MA, Duarte MA, Marques ME, Salvadori DM (2005d). *In vitro biocompatibility tests of two commercial types of mineral trioxide aggregate*. Pesquisa Odontologica Brasileira 19,183-187.

Ribeiro DA, Matsumoto MA, Duarte MA, Marques ME, Salvadori DM (2006c). Ex vivo biocompatibility tests of regular and white forms of mineral trioxide aggregate. *International Endodontic Journal* 39,26-30.

Ribeiro DA, Matsumoto MA, Marques ME, Salvadori (2007b). Biocompatibility of gutta-percha solvents using in vitro mammalian test-system. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* 103,106-109.

Ribeiro DA, Matsumoto MA, Padovan LE, Marques ME, Salvadori DM (2007d). Genotoxicity of corrosion eluates obtained from endosseous implants.*Implant Dentistry* 16, 101-9.

Ribeiro DA, Salvadori DM, Marques ME (2005e). Abnormal expression of bcl-2 and bax in rat tongue mucosa during the development of squamous cell carcinoma induced by 4-nitroquinoline 1-oxide. *International Journal of Experimental Pathology* 86, 375-381.

Ribeiro DA, Scolastici C, De Lima PL, Marques ME, Salvadori DM (2005a). Genotoxicity of antimicrobial endodontic compounds by single cell gel (comet) assay in Chinese hamster ovary (CHO) cells. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* 99,637-640.

Ribeiro DA, Scolastici C, Marques ME, Salvadori DM (2004a). *Fluoride does not induce DNA breakage in Chinese hamster ovary cells in vitro.* Pesquisa Odontologica Brasileira 18,192-196.

Ribeiro, DA, Marques MEA, Salvadori DMF (2006e). Genotoxicity and cytotoxicity of glass inomer cements on chinese hamster ovary (CHO) cells. *Journal of Materials Science Materials in Medicine* 17,495-500.

Ribeiro, DA, Sugui MM, Matsumoto MA, Duarte MAH, Marques MEA, Salvadori DMF (2006b). Genotoxicity and cytotoxicity of mineral trioxide aggregate and regular and white Portland cements on Chinese hamster ovary (CHO) cells in vitro. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* 101,258-261.

Rocha MEM, Ferreira AMDC, Bechara EJH (2000). Roles of phosphate and an enoyl radical in ferritin iron mobilization by 5-aminolevulinic acid. *Free Radical Biology and Medicine* 29, 1272-1279.

Sakagami Y, Yamazaki H, Ogasawara N, Yokoyama H, Ose Y, Sato T (1988). The evaluation of genotoxic activities of disinfectants and their metabolites by UMU test. *Mutation Research* 209,155-160.

Samuelsen JT, Dahl JE, Karlsson S, Morisbak E, Becher R (2007). Apoptosis induced by the monomers HEMA and TEGDMA involves formation of ROS and differential activation of the MAP-kinases p38, JNK and ERK. *Dental Materials*, 23, 34-39.

Sato M, Tsuchiya H, Kato M, Yamamoto K, Nakazato G, Takagi N, Namikawa I (1989). Effects of Tween 80 and sodium fluoride on extracellular glucosyltransferase production and membrane lipids of Streptococcus mutans. *International Journal of Biochemistry* 21,751-754.

Satoh R, Kishino K, Morshed SR, Takayama F, Otsuki S, Suzuki F, Hashimoto K, Kikuchi H, Nishikawa H, Yasui T, Sakagami H (2005). Changes in fluoride sensitivity during in vitro senescence of normal human oral cells. *Anticancer Research* 25,2085-2090.

Schweikl H, Schmalz G, Bey B (1994). Mutagenicity of dentin bonding agents. *Journal of Biomedical Materials Research* 28,1061-1067.

Schweikl H, Schmalz G, Federlin M (1998a). Mutagenicity of the root canal sealer AHPlus in the Ames test. *Clin Oral Investig.* ;2:125-9.

Schweikl H, Schmalz G, Rackebrandt K (1998b). The mutagenic activity of unpolymerized resin monomers in Salmonella typhimurium and V79 cells. *Mutation Research* 415,119-130.

Slamenova D, Gabelova A, Ruppova K (1992). Cytotoxicity and genotoxicity testing of sodium fluoride on Chinese hamster V79 cells and human EUE cells. *Mutation Research* 279,109-115.

Souza PP, Aranha AM, Hebling J, Giro EM, Costa CA (2006). In vitro cytotoxicity and in vivo biocompatibility of contemporary resin-modified glass-ionomer cements. *Dental Materials* 22, 838-44.

Spahl W, Budzikiewicz H, Geurtsen W (1998). Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. *Journal of Dentistry* 26,137-145.

Stea S, Visentin M, Cervellati M, Verri E, Cenni E, Savarino L, Stea S, Pizzoferrato A (1998). In vitro sister chromatid Exchange induced by glass ionomer cements. *Journal of Biomedical Materials Research* 40,545-550.

Tai KW, Huang FM, Huang MS, Chang YC (2002). Assessment of the genotoxicity of resin and zinc-oxide eugenol-based root canal sealers using an in vitro mammalian test system. *Journal of Biomedical Materials Research* 59,73-77.

Tomakidi P, Koke U, Kern R, Erdinger L, Krüger H, Kohl A, Komposch G (2000). Assessment of acute cyto- and genotoxicity of corrosion eluates obtained from orthodontic materials using monolayer cultures of immortalized human gingival keratinocytes.*Journal Orofacial Orthopedics* 61, 2-19.

US Environmental Protection Agency (USEPA) (2007). Guidelines for mutagenicity risk assessment. Avaliable from *http://www.epa.gov/* (Assessed June, 1, 2007).

Velazquez-Guadarrama N, Madrigal-Bujaidar E, Molina D, Chamorro G (2005). Genotoxic evaluation of sodium fluoride and sodium perborate in mouse bone marrow cells. *Bulletin of Environmental Contamination and Toxicology* 74,566-572.

Voegel E (1973). Strong antimutagenic effects of fluoride on mutation induction by trenimon and 1-hyenyl-3,3-dimethyltriazene in Drosophila melanogaster. *Mutation Research* 20,239-252.

Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, Yang KD (2004). Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. *Biomedical Environmental Science* 17,217-222.

Warner RR, Myers MC, Burns J, Mitra S (1993). Analytical electron microscopy of chlorhexidine-induced tooth stain in humans, direct evidence for metal-induced stain. *Journal of Periodontal Research* 28,255-265.

Wilson AD, Kent BE (1972). A new translucent cement for dentistry. The glass ionomer cement. *British Dental Journal* 132,133-135.

Woolverton CJ, Haywood VB, Heymann HO (1993). Toxicity of two carbamide peroxide products used in nightguard vital bleaching. *American Journal Dentistry* 6, 310-4.

World Health Organization (WHO). Available from URL, *http,//home.iae.nl/users/lightn.et/health/fluoride.htm. 1994*.

Wtihrow TJ, Brown NT, Hitchins VM, Strickland AG (1989). Cytotoxicity and mutagenicity of ophtalmic solution preservatives and UVA radiation in L5178Y cells. *Photochemistry Photobiology* 50,385-89.

Zhang M, Wang A, He W, He P, Xu B, Xia T, Chen X, Yang K (2007) Effects of fluoride on expression of NCAM, oxidative stress, and apoptosis in primary cultured hippocampal neurons. *Toxicology*236:208-16.

Zhang M, Wang A, Xia T, He P (2008) Effects of fluoride on DNA damage, S phase cell cycle arrest and expression of MF-kB in primary culture rat hippocampal neurons. *Toxicology Letters*179: 1-5.

Zouain-Ferreira SL, Zouain-Ferreira TR, da Silva CR, Cervantes Dias KR, Caldeira-de-Araujo A, Bernardo-Filho M (2002). Radiation induced-like effects of four home bleaching agents used for tooth whitening, effects on bacterial cultures with different capabilities of repairing deoxyribonucleic acid (DNA) damage. C*ellula*r *and* Molecular *Biology* (Noisy-le-grand) 48,521-524.

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