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FRONTIER

Risk aspects of dental restoratives: From amalgam to tooth-colored materials

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Abstract

Dental materials' choice of patients has considerably changed. Whereas cast gold and amalgam have been the predominant biomaterials for decades, today tooth-colored materials like resin-based composites and ceramics are more and more successful. However, are we going to replace a good but biologically questionable material (amalgam) with an equal material (resin composite) being more esthetic but also biologically questionable? For amalgam, long-term clinical studies reported some significant hints that in single cases amalgam may be a health hazard for patients, finally Norway banned amalgam completely. The main advantage of a resin-based composite over amalgam is its tooth-like appearance and more or less absence of extensive preparation rules. For many years it was believed that resin-based composites may cause pulpal injury. However, pulpal injury associated with the use of resin-based composites is not correlated with their cytotoxic properties. Nevertheless, resin-based composites and other dental materials require rigorous safety evaluation and continuous monitoring to prevent adverse events similar like with amalgam. Because of non-biocompatible pulp responses to resin-based composites and amalgam, they should not be placed in direct contact with the dental pulp. The less dentin remaining in the floor of preparations between resin-based composites or other dental materials is more likely to cause pulpitis. Percentage of patients and dental practitioners who display allergic reactions is between 0.7% and 2%. The release of cytotoxic monomers from resin-based materials is highest after polymerization and much lower after 1 wk. Substances released from resin-based composites have been shown to be toxic in cytotoxicity tests. Nevertheless, *in vitro* cytotoxicity assays have shown that amalgam has greater toxic effects than resin-based composites, sometime 100-700-fold higher. Altogether, the risk of side-effects is low, but not zero, especially for dental personnel.

Key words: Exposures; Restoratives; Amalgam; Resin-based composites; Adhesives

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INTRODUCTION

The choice of dental materials has considerably changed

during the last 20 years[1-3]. In former times, cast gold and amalgam have been the materials of choice for decades[4]. However, after amalgam was alleged to be inacceptably toxic and simultaneously esthetic demands of patients were growing, tooth-colored materials like resin-based composites and ceramics took more and more parts of this huge market[5].

In terms of biocompatibility and exposure, cast gold may be still the best restorative material, however, it is non-esthetic when it is used in visible areas such as premolars and also here some health concerns in terms of gold allergies are present[6]. Furthermore, the gold prize considerably increased from < $ 200 to > $ 1000 per ounce during the last decade which is consequently also transferred to restoration costs and therefore being detrimental for cost effectiveness as well. Other highly biocompatible materials like phosphate or glass ionomer cements are too brittle and therefore not able to withstand intraoral occlusal forces in deciduous and permanent teeth over time[1,2].

Today's restorative trend clearly answers the question "black or white?" by more and more moving from metallic amalgam to resin-based composites[5]. The same is true for bonded all-ceramic restorations such as ceramic inlays and onlays, because they have to be adhesively luted with the same adhesives and resin-based composite luting cements. So we face the interesting question whether we are replacing a clinically good but biologically questionable material (amalgam) with an equal material being more esthetic (resin composite) but also (or even more?) biologically questionable.

AMALGAM

Amalgam is one of the by far most successful dental restoratives which has been used all over the world since more than 150 years[7-10]. Long-term data are sufficient and long-term costs due to repair, refurbishment and tooth hard tissue loss during replacement are favourable[1-3,11-15]. Disadvantages are a compromised esthetic appearance due to an argentic to black color and especially biocompatibility concerns[7,16-24]. Dental silver amalgam consists of 50% mercury (in a complex mixture of copper, tin, silver, and zinc) and therefore this material was always suspected to be a considerable hazard for both patient and environment[25-35].

In the literature of the field, two opposing groups are identified: Primarily toxicologists are arguing against the health risks of mercury vapor being released from amalgam restorations and potentially threatening health of both patients and dentists and moreover polluting the environment by dental mixing and application processes[25-50]. On the other hand many authors with clinical dental background repeatedly state that amalgam per se is one of the most successful restorative materials[3,5,8,11,13,14,23,24,51-65]. So what is the real threat with amalgam? It is common knowledge that high-dose exposure to elemental mercury vapor cause several diseases like emotional dysfunction[53]. However, it is not fully understood to the date whether smaller amounts like being released from amalgam restorations are a considerable health hazard as well[51-53,66,67].

In a retrospective cohort study involving 20 000 participants over 20 years (1977-1997) in the New Zealand defense force[28]. The cohort was linked with morbidity records by use of a time-varying exposure unit of 100 amalgam surface-years. Multiple sclerosis had an adjusted hazard ratio of 1.24, but there was no association with chronic fatigue syndrome (0.98), or kidney diseases[28]. Also Aminzadeh *et al*[25] reported some hints for a possible correlation of amalgam restorations and multiple sclerosis, however, also stating that more clinical studies are needed.

One of the most intensive clinical trials so far was the New England's Children Amalgam Trial (NECAT) giving clinical result. 534 children (6-10 years old) with carious primary molars received either amalgam or resin composite restorations. Evaluated parameters were neuropsychological outcome (Full-Scale IQ score, General Memory Index, Visual-Motor Composite of the Wide Range Assessment of Visual Motor Abilities) and renal glomerular function with no statistical differences between resin composite and amalgam groups in any of the investigated criteria[51,52,68]. Furthermore, parent-completed child behavior checklists and children's self-reports were collected. Children's psychosocial status was evaluated in relation to three indices of mercury exposure: treatment assignment, surface-years of amalgam, and urinary mercury excretion. Again, there was no evidence that exposure to mercury from dental amalgam was associated with adverse psychosocial outcomes[53]. In another part of NECAT, longitudinal amalgam exposure data in children randomized to amalgam restorations were analyzed. Amalgam and U-Hg were moderately correlated with the total of amalgam surfaces having been a good predictor of current U-Hg and posterior occlusal surface-years for cumulative U-Hg. One additional amalgam surface caused a 9% increase in current U-Hg, and one posterior occlusal surface-year resulted in a 3% increase in cumulative U-Hg excretion[61]. Finally it could be shown that daily chewing gum use resulted in higher urinary Hg levels[69].

Halbach *et al*[56] measured internal exposure to amalgam-related mercury in plasma and erythrocytes after amalgam removal and estimated the amalgam-related absorbed dose in 82 patients. Post-removal steady-state Hg concentrations were taken for 18 mo for three groups: Removal of the fillings/removal and non-specific detoxification/health promotion program without removal. After amalgam removal, inorganic Hg was decreased, leveling at 27% of pre-removal levels after 60 d. Organic Hg in plasma did not change. Organic Hg in red cells of group A was lower in the early post-removal phase and higher in the late post-removal phase, being higher than the pre-removal control. A protracted increase in organic Hg was also found in red cells of group B after 60 d. In all groups, time profiles of urinary concentra-

tion and excretion of total-Hg were similar to those of inorganic-Hg levels in plasma. It was estimated that the amalgam-related inhalation and ingestion of Hg species were within the limits proposed by the World Health Organization (WHO), Agency for Toxic Substances and Desease Registry, and Environmental Protection Agency. The integrated daily Hg dose absorbed from amalgam was estimated < 3 μg for an average number of fillings and 7.4 μg for high amalgam load, with 30 μg being the tolerable dose according to the WHO[56].

On the other hand, there is no doubt that amalgam restorations release small amounts of mercury during clinical service which is absorbed by several body tissues in human subjects[32-35,64,70-75]. The daily dose is found to be 14% of the threshold above which observable adverse neurological symptoms are expected[75]. It has reported that methyl mercury and inorganic mercury levels in blood and cortex of autopsy bodies with a significant correlation between methyl mercury in blood and occipital cortex. Inorganic mercury in blood and occipital cortex, as well as total-Hg in pituitary and thyroid were strongly associated with the number of dental amalgam surfaces at the time of death[46,67]. Mutter *et al*[33] repeatedly stated that some of the clinical studies reporting low to no risk connected with dental amalgam may be methodically flawed which may lead to inadequate conclusions about the safety of dental amalgam. He also identified mercury vapor as potential reason for autism or Kawasaki's disease[34,35,76]. It is also controversially discussed whether carbamide peroxide tooth bleaching agents lead to an increased release of mercury[77,78].

Another important point in the amalgam issue is occupational exposure for dentists and dental nurses[26,31,79-87]. It was found that a correlation between total Hg-U and duration of dental practice exists[87]. However, a cytogenetic damage in oral health professions dealing with amalgam was not reported[26,87]. Farahat *et al*[82] showed that dental staff have significant exposure to mercury vapor, furthermore indicating a negative impact of mercury on thymus gland functions[82,87]. Jones *et al*[84] investigated possible residual adverse effects from occupational mercury exposure in dentistry in 115 graduates of a dental nurse school from 1968-1971 because 30 years ago, dental nurses worked with amalgam without protective gloves or a ventilation system, resulting in chronic mercury exposure. Significant differences were found in current health experience and reproductive health, especially early hysterectomy experience. Reporting of Occupational Overuse Syndrome was strongly positively correlated with years of work.

Finally, also environmental aspects of mercury pollution by amalgam waste of dental practices and clinics have to be considered. Mercury occurs in nature as sulfides and in some minerals. All over the world every year 20 000-30 000 tons of mercury are discharged into the environment. Less than 50% of freshly triturated amalgam is inserted in cavities, more than 50% is waste. Extracted teeth with preexisting amalgams, amalgam-contaminated capsules and cotton rolls are discharged with the solid waste. However, dental mercury contamination makes only 3%-4% of global mercury being insignificant compared with industrial pollution[30,80,88,89]. With proper amalgam separators it could be even more reduced[30,80,88,89].

Despite all hints towards side-effects caused by mercury vapor of dental amalgam restorations, unproportionally many patients suffer amalgam incompatibility. Gottwald *et al*[55] conducted an interdisciplinary case-control study with special focus on toxicological, allergic, psychological and psychiatric aspects. Patients with amalgam-associated complaints (n = 40) were compared to amalgam bearers without complaints (n = 40) regarding quantity, surface area and quality of amalgam fillings, mercury load in blood and urine, allergy examination, and psychometric assessment with questionnaires noting coping strategies, interpersonal problems and self-consciousness. Patients and controls did not reveal different mercury concentrations in body fluids with patients having higher levels of psychic distress, higher incidence of depression and somatization disorders as well as different styles of coping with anxiety compared to controls. So the theory of amalgam-related complaints as an expression of underlying psychic problems was confirmed. A socio-economically important issue is that a ban of dental amalgam would also have some economic impact. Beazoglou *et al*[90] calculated the economic costs of an amalgam ban in the United States with total expenditures for restorations increasing from $ 46.2 billion to $ 49.7 billion and with consequently 15 444 021 fewer restorations inserted per year. An estimated first-year impact of an amalgam ban means an increase in expenditures of $ 8.2 billion.

Altogether it can be summarized that long-term clinical studies primarily demonstrated that amalgam can be safely used for patients, dental staff, and environment. However, there are some significant hints that in single cases amalgam may be a health hazard for patients. From 2008, Norway banned amalgam completely which is another hint[58]. So, amalgam remains an excellent restorative material with centuries of clinical success and decades of significant problems in biocompatibility.

RESIN-BASED COMPOSITES

A remarkable change in restorative dentistry has been the dramatic drop in the use of amalgam to restore teeth[91]. Patient and practitioner demand for a tooth-colored material as an alternative to amalgam was addressed in 1955 by Dr. Buonocore who described the use of a plastic material to restore teeth[92]. Later in 1950s, the first tooth-colored direct restorative material called Sevitron was produced by L.D. Caulk[93]. In the 1960s, several resin-based composite dental restorative materials (resin-based composites) were introduced[94]. The main advantage of a resin-based composite over amalgam is that it can be made in a wide range of tooth colors allowing the almost invisible restoration of teeth. However, the benefits of

resin-based composites in comparison with amalgam and other dental materials have proved to be controversial. Normally resin-based composites can be used to restore teeth and repair or replace failing restorations with less removal of vital tooth structure in comparison with amalgam[95].

Unlike amalgam, resin-based composites must be bonded to teeth using an adhesive, which makes them more expensive and more technique-sensitive. Without meticulous placement, resin-based composite restorations can fail quickly. Nevertheless, even with the most meticulous placement, the longevity of resin-based composite restorations placed in posterior teeth has been shown to be significantly less than amalgam restorations[96]. The main reasons for the inferior clinical longevity of composite restorations in comparison with amalgam are marginal discoloration and a loss of adhesion[97].

Resin-based composites shrink by approximately 5% upon light-curing, which can create gaps for bacterial microleakage along the cavity margins[98]. These examples indicate that many of the problems patients have suffered with resin-based composites does not appear to be directly caused by the chemicals within the formulation of the material, but because of the shortcomings of the material when it is used to restore teeth. The shortcomings of resin-based composites, particularly their polymerization shrinkage, are an active area of research and new lower shrinkage materials are under development to help improve their clinical performance similar to amalgam restorations.

The earliest resin-based composites had the worst longevity because they were prone to breakage and leakage due to their weak compressive strength[99]. The initial techniques to etch enamel to bond dental restorations were also not very successful, so many restorations suffered a loss of adhesion and were lost[100]. Many clinicians were initially reluctant to bond to dentin because they feared the high acid content of the etchant would cause a necrosis of underlying pulp tissue[101]. Subsequently, it was discovered that the buffering capacity of dentin, along with an improved quality of sealing to reduce microleakage, reduced the pulp irritation beneath resin-based composite restorations[102]. As research progressed, the concept of the "hybrid layer" was created to explain the physical and chemical interactions of the adhesive, resin-based composite, and tooth structure[103]. The "hybrid layer" concept has proved to be useful to develop research strategies to increase the quality of sealing and bonding of resin-based composites to tooth structure[104]. Improvements to the process of accomplishing resin-based composite bonding to tooth structure progressed through a number of "generations". Each new generation of resin-based composite materials have had improved bonding and physical properties which are beneficial to patients through their increased longevity[105]. The current, 7th generation of resin-based composite adhesives can accomplish very high bond strengths to tooth structure[106]. The newest generations of "one-step" resin-based composite materials are generally easier for practitioners to use, and help reduce the exposure of patients to failed restorations.

For many years it was believed that the toxicity of the chemicals in the resin-based composite materials was responsible for pulpal injury. However, pulpal injury associated with the use of resin-based composites could not be correlated with their cytotoxic properties[107]. The discovery of the effect of bacterial contamination on the vitality of the tooth pulp, was a major milestone in dental research. In general, resin-based composites and other dental materials do not provide a hermetic seal with the tooth structure. Bacterial leakage may subsequently occur. The presence of bacteria and their toxic products can evoke an inflammatory response in the underlying pulp. Suh *et al*[108] demonstrated that the growth of bacteria in cavity restorations was directly correlated with pulpal inflammatory responses in the adjacent pulp tissue. As yet no permanent filling material has shown to consistently provide a perfect marginal seal, so leakage and bacterial contamination are always a threat to the integrity of the pulp. Therefore, the antibacterial properties of restorative materials are of considerable importance, and this explains the clinical success of some cytotoxic restorative materials, such as zinc oxide eugenol[109]. Despite these findings, it must be acknowledged that generally it is preferable to use dental materials which have the least potential to be toxic to patients and dental professionals. Similar to amalgam, resin-based composites and other dental materials require rigorous safety evaluation and continuous monitoring[110] to prevent adverse events.

Dentin and enamel have different physical properties and elemental compositions which have complicated the resin-based composite bonding to tooth structure[111]. It was discovered that the inclusion of hydrophobic monomers in adhesives could not penetrate the aqueous environment of demineralized dentin. Thus, methacrylate-based priming agents were used to create a permeable interface for the formation of a hybrid layer[112] which can increase micromechanical retention of the resin-based composite[113]. Thus, the need for "wet bonding" arose, and techniques for preparing the interface for increasingly hydrophobic monomers were developed[114]. Wet bonding systems have been successful[115]. However, they require the handling of multiple components which must be used in multiple steps. To facilitate the ease and speed with which bonding can be accomplished, the latest generation of "one-step adhesive systems" have been introduced which don't have a separate acid etching step. Instead, acrylic resin monomers themselves provide the acidity needed for demineralization and simultaneously penetrate exposed and uplifted collagen fibrils[116]. A dental composite typically consists of a resin-based oligomer matrix, such as a bisphenol A-glycidyl methacrylate (Bis-GMA) or urethane dimethacrylate (UDMA), and an inorganic filler such as silicon dioxide silica. Compositions vary widely, with proprietary mixes of resins forming the matrix, as well as engineered filler glasses and glass

ceramics. The filler gives the composite wear resistance and translucency. A coupling agent such as silane is used to enhance the bond between these two components. An initiator package (such as: Camphorquinone, Phenylpropanedion or Lucirin) begins the polymerization reaction of the resins. A catalyst is added in varying concentrations to control the speed of polymerization[117]. Resin-based composite materials are all capable of causing moderate to severe cytotoxicity when placed in contact with *in vitro* cell lines[118]. Resin-based composite materials may also cause severe pulp necrosis when used for direct-pulp capping[119]. The migration of adhesive and resin-based composite particles into pulp tissue can stimulate inflammatory responses[120]. Because of these non-biocompatible pulp responses to resin-based composites and amalgam, they should not be placed in direct contact with the dental pulp. A biocompatible liner such as $Ca(OH)_2$ or preferably; mineral trioxide aggregate (MTA) must be used as a liner to help prevent unfavorable responses to direct pulp capping with resin-based composite[121] or amalgam. An MTA or $Ca(OH)_2$ liner is not needed in shallow indirect pulp capping restorations because the buffering effect of dentin can prevent the diffusion of chemicals from resin-based composites and amalgam from entering the pulp tissue, particularly when the dentin thickness is above 0.5 mm[122]. The less dentin remaining in the floor of preparations between resin-based composites or other dental materials is more likely to cause pulpitis[122].

A number of local and systemic reactions to resin-based composite materials have been reported. The incidence of patients and dental practitioners who display allergic reactions is between 0.7% and 2%[123-126]. The main source of cellular and molecular cytotoxic injury from resin-based materials is claimed to be the leaching of unpolymerized monomers from the restoration during and after polymerization[127] which can reduce pulp vitality and cause a retraction of the gingival margin[128,129]. The release of cytotoxic monomers from resin-based materials is highest after polymerization and much lower after 1 wk[130]. Which may suggest the health risks to patients and practitioners are highest when in contact with newly polymerized resin-based composite materials, and the health risk diminishes over time.

Erosion and saliva degradation of resin-based composites may cause the release of leachable substances. Human-saliva derived esterases can biodegrade resin-based composites, causing the release of (Bis-GMA) monomers and (UDMA-type) comonomer[131]. The substances released from resin-based composites, particularly the (Bis-GMA) monomers have been shown to be toxic in cytotoxicity tests[132]. The presence of leached compounds is dependent on the formulation of resin-based composite[133]. The more flowable resin-based composites are more toxic than the traditional resin-based composites[134]. The relative *in vitro* cytotoxicity of resin-based composite monomers measured using a bromodeoxyuridine assay discovered that the Hg^{2+} amalgam component was four-fold more toxic than Bis-GMA to human gingival fibroblasts[135]. Almost all the *in vitro* cytotoxicity assays have shown that amalgam has greater toxic effects than resin-based composites, sometime 100-700-fold higher[136]. A problem is the general lack of resin-based composite biocompatibility data in comparison with amalgam. The results from systemic toxicity tests of resin-based composites do not indicate any unacceptable risk to the patient's general health[137]. The *in vitro* screening of some components of resin-based composites are mutagenic[138]. Due to the limitations of the *in vitro* genotoxicity test systems and the comparatively high concentrations needed to elicit the reactions, no unacceptable risk can yet be derived from those data for the patient[139]. Most of the available data suggests that amalgam is relatively more hazardous to patients and dental professionals, than resin-based composites.

Skin and mucosa which come into contact with resin-based composites and bonding agents can become slightly inflamed which is commonly observed as a reddening of the affected area. However, if a patient or dental professional is allergic to a compound within the resin-based composite their reactions may be more severe and allergic irritant contact dermatitis can be observed. Contact urticaria, pigmentary changes, and photoallergic contact dermatitis may occasionally occur. Rarely other health effects, such as respiratory and neurologic signs and symptoms have been reported, but none have been linked to dental resin-based composites[140]. The concentrations are probably too minute to cause systemic reactions[137]. The most common resin-based composites to cause contact dermatitis, are (meth)acrylics, polyurethanes, phenol-formaldehydes, polyesters, amino resins (melamine-formaldehydes, urea-formaldehydes), polyvinyls, polystyrenes, polyolefins, polyamides and polycarbonates[140]. Contact dermatitis usually presents on the hands, fingers, and forearms, while facial, eyelid, and neck involvement may occur through indirect contact, *e.g.*, *via* the hands, or from airborne exposure[140]. Patch testing with commercially available materials is important for a diagnosis of an allergy[141]. In some countries, occupational dermatoses are relatively common among dental staff, sometimes entailing occupational disability and re-schooling[142]. The risk of occupational dermatoses can be reduced by the development of new bonding techniques and careful risk-benefit assessments in the formulation of new dental composites. To protect patients from potential hazards of light-cured monomers released from resin-based composites it is important to use an effective curing unit and to applying the light-curing for the recommended length of time[142]. To protect dental professionals from the potential hazards of monomers released from resin-based composites, gloves should always been worn to prevent direct skin contact.

THE RISK ASPECT IN RESTORATIVE DENTISTRY

Dental restorative materials represent the most frequent

replacement materials in the human body[143]. Despite that fact, biocompatibility issues regarding dental materials (especially amalgam) have not been scientifically evaluated until the early 1980s[141]. During the last two decades, however, amalgam lost its unique feature because adhesively bonded resin composites got suitable even for stress-bearing posterior restorations[144]. The paradigm shift towards minimally invasive restorations additionally supported this trend[145]. However, in many cases there is almost no patient or dental staff knowledge of hazards by the use of dental restoratives[146]. Furthermore it is of significant interest whether recently used dental materials changed the use-risk ratio.

Fundamental judgement tool of dental materials is a risk analysis. Schmalz *et al*[145] defined the term "risk" concerning biocompatibility of dental restoratives as "the probability of a side effect and the severity of that side effect". Risk analysis implies the description of indication ranges of a medical product, analysis of tissue exposure, and potential hazard[147]. So risk analyses try to determine the probability and severity of side effects for human health by exact knowledge of their composition. The consecutive risk assessment clarifies under estimation of usefulness and risk, whether a medical product may enter the market. Here it is decisive to compare the advantages of the material with the frequency and severity of side-effects[148]. In restorative dentistry, primarily a potential hazard by release of ingredients is discussed. Dental biomaterials are medical products with medium hazard potential. This means that clinical investigations are not mandatory in Europe, manufacturers just have to meet minimum requirements[147]. Especially in the post-amalgam era in the middle of the 1990s, some restoratives diminished from the market because minimum requirements were not achieved (Figure 1)[149].

Systematic epidemiological studies concerning frequency of side-effects with dental biomaterials are missing. Mjör[147] reported possible side-effects with different materials with 13 325 sessions (done by 137 dentists) and 24 cases of subjective discomfort, 7 cases of acute nature, and 15 cases of long-standing effects. In eight cases, amalgam was the reason for patients' complaints[150]. So altogether the risk of side-effects is low, but not zero[148].

Dental personnel is much more under risk than patients. Geukens *et al*[148] observed 13 000 patients with contact dermatitis. In 31 patients (meth) acrylates were responsible for the complaints, and almost 50% of these group was working as dentist or dental nurse or dental technician[151]. Unfortunately, latex or vinyl gloves do not guarantee for safety due to their permeability at least after some minutes. This should be one of the reasons that dental personnel reveal increased rates of contact dermatitis of the fingers or allergic reactions following contact with monomers[152-154]. Thus, it is clearly recommended to completely avoid contact with unpolymerized resins[147].

Side-effects of dental materials are primarily of a local nature (*e.g.*, gingivitis, mucosal alterations, pulpitis, *etc.*) or allergic (type I : immediate reaction or type IV: delayed reaction). Contact allergies have been observed for nickel

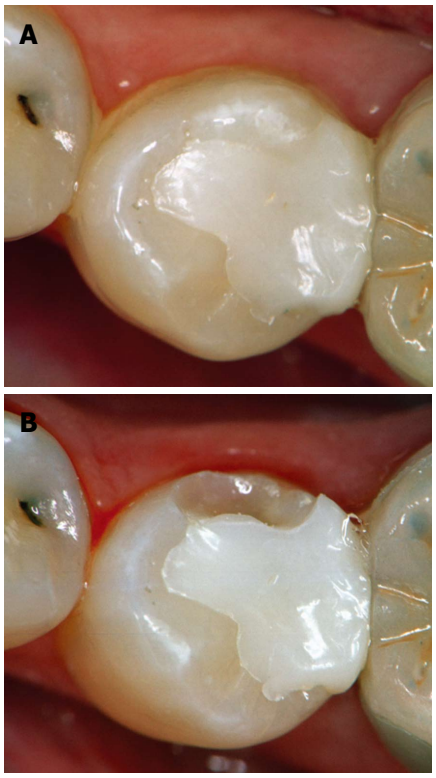

Figure 1 Some restoratives diminished from the market. A: Ariston restoration in lower second premolar at baseline; B: Due to a 2% linear expansion, 18 mo of clinical service were enough to disrupt the lingual cusp.

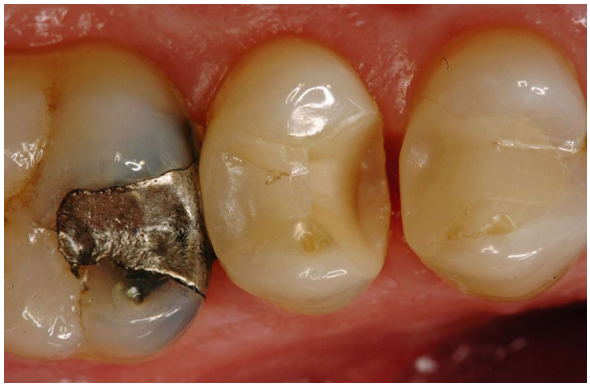

Figure 2 Fracture of a ceramic inlay in a upper first premolar.

sulfate, potassium dichromate, cobalt chloride, palladium chloride and gold sodium thiosulfate in patients with presence of metal allergy[155].

Other systemic effects (*e.g.*, mutagenic, cancerogenic or teratogenic) are more of a theoretical nature[147]. Although there is a proven amount of substance release, this does not automatically mean an inacceptable health hazard[137]. Bacsik *et al*[156] could show estrogenic effects by bisphenol-A in mice, however, it is a matter of clinical relevance when substances are directly injected into the stomach of the animals instead of investigating true release from restorations. So also here clinical studies remain the ultimate instrument for risk assessment[157]. In the course of prospective clinical studies with dental

biomaterials, the risk aspect plays a minor role. Main focus here are longevity aspects such as marginal integrity, restoration integrity, hypersensitivities, and recurrent caries. Moreover, patient numbers in dentistry *e.g.*, for studies with posterior restorations are normally in the range of 30 patients[158] which may not be of sufficient power to describe side-effects. This is clearly reflected by evaluations of side-effects with local anesthesia. Despite 0.5 million local anesthetic injections which are administered in the United States daily, the actual risks of toxicity from these local anesthetic injections remain more or less unknown[159]. Therefore, prospective clinical studies mainly concentrate on local risks such as pulp reactions, compatibility with gingiva/periodontium, irritation of the oral mucosa, or biofilm accumulation[157].

The benefit of dental biomaterials is still related to longevity. Kaplan-Meier survival curves and the associated nonparametric log rank test statistic are methods of choice for estimation of survival and therefore also failure risk[159]. This risk is appropriately reflected by annual failure rates[160,161]. Quality assessment of dental restorations is carried out according to modified USPHS critera with clinical examinations and analysis of replicas[162]. Main failure reasons are related to crucial criteria "marginal quality", "restoration integrity", "tooth integrity", and "hypersensitivities" (Figure 2)[162]. Concerning clinical success, ADA criteria of 1996 are still valid. Failure rates < 10% after 4 years are defined as acceptable.

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BRIEF ARTICLE

Effects of low intensity laser irradiation phototherapy on dental pulp constructs

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Abstract

AIM: To investigate low intensity laser irradiation phototherapy (LILIP) on the proliferation, mineralization and degradation of dental pulp constructs.

METHODS: Stem cells from human exfoliated deciduous teeth (SHED) were grown to confluence and seeded on collagen scaffolds to create dental pulp constructs. LILIP was delivered to the dental pulp constructs using an 830 nm GaAlAs laser at an output power of 20 mW. The LILIP energy density was 0.4, 0.8, 1.2, and 2.4 J/cm^2. After 8 d, the cell proliferation and degradation within the dental pulp constructs were measured using histologic criteria. After 28 d, the effect of LILIP on SHED mineralization was assessed by von Kossa staining.

RESULTS: SHED proliferation within the dental pulp constructs varied after exposure to the 0.4, 0.8, 1.2, and 2.4 J/cm^2 LILIP energy densities ($P < 0.05$). The maximum proliferation of SHED in nutrient deficient media was 218% after exposure to a 1.2 J/cm^2 LILIP energy density. SHED grown in nutrient deficient media after exposure to a 0.4, 0.8, and 1.2 J/cm^2 LILIP energy density, proliferated by 167-218% compared to the untreated (non-LILIP) control group ($P < 0.05$). SHED exposed to a 0.4, 0.8, and 1.2 J/cm^2 LILIP energy density, and grown in optimal nutritional conditions and proliferated by 147%-164% compared to the untreated (non-LILIP) control group ($P < 0.05$). The exposure of SHED to the highest LILIP energy density (2.4 J/cm^2) caused a reduction of the cell proliferation of up to 73% of the untreated (non-LILIP) control ($P < 0.05$). The amount of mineral produced by SHED increased over time up to 28 d ($P < 0.05$). The 0.8 and 1.2 J/cm^2 LILIP energy densities were the most effective at stimulating the increased the mineralization of the SHED from 150%-700% compared to untreated (non-LILIP) control over 28 d ($P < 0.05$). The degradation of dental pulp constructs was affected by LILIP ($P < 0.05$). The dental pulp constructs grown in optimal nutritional conditions exposed to a 0.8 J/cm^2 or 1.2 J/cm^2 LILIP energy density had 13% to 16% more degradation than the untreated (non-LILIP) control groups ($P < 0.05$). The other LILIP energy densities caused a 1% degradation of dental pulp constructs in optimal nutritional conditions ($P > 0.05$).

CONCLUSION: LILIP can enhance or reduce SHED proliferation, degradation and mineralization within dental pulp constructs. LILIP could promote the healing and regeneration of dental tissues.

Key words: Dental pulp cells; Proliferation; Low intensity laser; Low intensity laser irradiation phototherapy; Stem cells from human exfoliated deciduous teeth

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INTRODUCTION

Regenerative endodontics procedures are biologically based procedures that are used to replace the damaged dentin and root structures of teeth as well as cells of the pulp-dentin complex[1]. Regenerative endodontic procedures are: root canal revascularization, apexogenesis, apexification, partial pulpotomy, direct pulp capping, stem cell therapy and dental pulp constructs[1]. Endodontic regenerative procedures are widely expected to become more common in coming decades[2]. The increased usage of regenerative therapies is likely because of the discovery of dental stem cells, the use of improved treatment protocols, and the availability of new technologies[1]. The success of regenerative endodontic procedures is dependent on stimulating the proliferation and mineralization activity of stem cells from human exfoliated deciduous teeth (SHED) and other dental stem cells[3]. Previous research has demonstrated pulp healing and regeneration by adding growth factors to increase dental pulp stem cells (DPSCs) activity[4]. No previous research has investigated the possibility of using lasers to increase SHED proliferation, mineralization or degradation of scaffolds.

Lasers are beneficial for some dental treatments, such as oral surgery[5], endodontics[6], periodontology[7], and restorative dentistry[8]. Low intensity laser irradiation phototherapy (LILIP) can change cell activity[9]. LILIP has been used in the treatment of dentin hypersensitivity, gingivitis, periodontitis, and to heal oral ulcers[10,11]. In response to LILIP, fibroblast cells can increase their rate of proliferation by 300% to 600%[12]. In response to LILIP, epithelial cells cultured in a nutritionally deficient state can dramatically increase their rate of proliferation[13]. LILIP can be effective in stimulating the proliferation and mineralization activity of osteoblasts and fibroblasts[12-14]. LILIP can increase the proliferation of DPSCs, as indicated by measuring their cell mitochondrial activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[15]. Regenerative endodontic procedures require SHED proliferation, mineralization and degradation of scaffolds if they are delivered into teeth as a dental construct[16,17] to attempt to regenerate teeth. However, the SHED responses to LILIP have not been evaluated. Consequently, there is a need to investigate the effects of LILIP on SHED proliferation, mineralization, and scaffold degradation, to identify its optimal and injurious effects, prior to its potential use as part of future regenerative endodontic procedures. The aim of this research was to investigate the effects of LILIP on the proliferation and mineralization SHED, and the degradation of dental pulp constructs.

MATERIALS AND METHODS

Cell cultures

The SHED was donated under a material transfer agreement with the National Institutes of Dental and Craniofacial Research (Bethesda, MD). Rat fibroblast L929 cells (ATTC, Manassas, VA) were used as a control treatment group cell line. The SHED were cultured in Dulbecco's modified Eagles medium (DMEM; BD Biosciences, Franklin Lakes, NJ) supplemented with 10% or 2.5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% gentamycin and amphotericin antibiotic supplement. Cell cultures were maintained at 37 ℃ in a humidified atmosphere of 5% CO_2 with the culture media being replenished every second day. Confluent cultures of SHED were collected by trypsinization (0.25% trypsin/EDTA; Mediatech, Inc., Herndon, VA).

Preparation of three-dimensional scaffolds for cell culture

Three-dimensional collagen scaffolds (Collacote; Zimmer Dental, Carlsbad, CA) were cut into 2 mm × 2 mm sheets. Each scaffold was soaked in Hanks' balanced salt solution (HBSS; Cellgro, Herndon, VA) and stored at 4 ℃. Before use, the HBSS was replaced by culture medium. The scaffolds were incubated in DMEM at 37 ℃ for 30 min before application of the cells to equalize culture conditions and temperature between the scaffolds and cells.

Creation of dental pulp constructs

The SHED were added to the scaffolds in fresh aliquots; each scaffold was seeded with a million ($\times 10^6$) cells to create dental pulp constructs[16,17]. The L929 ($\times 10^6$) cells were also applied to the scaffolds as a control treatment. The scaffolds were maintained in 6-well culture plates (BD Biosciences, Franklin Lakes, NY) containing 5 mL of culture media. The DMEM culture media was removed and replenished every 2 d. After 8 d of cell culture, the dental pulp constructs were transferred to 96-well plates.

Laser irradiation of dental pulp constructs

Laser irradiation was delivered with Gallium-Aluminum-Arsenide (GaAlAs) laser (Asah Medico Uni-Laser, Hvidovre, Denmark). The irradiations were performed in contact with the plate base using the punctual irradiation mode in a 0.252 cm^2 area[15]. The 830 nm laser was applied with output power setting of 20 mW. The laser device was calibrated using laser power meter (Model OPM-572; Sanwa, Tokyo, Japan). The clear base of a well from 96-well plate was separated and the laser power output was measured after the laser passed through the base to determine the exact energy density on the cells. This measurement was repeated three times and the aver-

age was calculated. The energy density was applied using 6, 12, 18, and 36 s of irradiation time. The energy density was calculated using the following formula: energy density (J/cm^2) = [power (W) × time (s)]/area (cm^2).

Vital staining of SHED in dental pulp constructs

During the cell culture of the dental pulp constructs, 0.0016% neutral red dye (JT Baker, Phillipsburg, NJ) cell viability marker was added to the DMEM in order to stain the vital cells dark red[1]. The SHED and L929s were cultured for 8 d on the collagen scaffolds, with 10 culture replicates for each of the constructs treatments. For each cell type, the experimental groups were: lased 6 s with 0.4 J/cm^2, lased 12 s with 0.8 J/cm^2, lased 18 s with 1.2 J/cm^2, and lased 36 s with 2.4 J/cm^2. The control for this investigation was including constructs without irradiation.

Histology of dental pulp constructs

The dental pulp constructs were removed from cell culture and fixed by submerging them in a 10% neutral-buffered formalin (BDH Chemicals, Poole, United Kingdom) solution for 24 h. All the tissue constructs were then dehydrated in a graded series of alcohols from 70% to 100%. The constructs were then embedded in paraffin wax blocks and cut into serial histologic sections of 5-μm thickness using a microtome. The histology sections were then mounted onto glass slides and covered with a cover slip using adhesive.

Pathohistometric analysis of tissue constructs

The numbers of stained neutral red SHED were counted as the number of vital metabolically active cells within each of the dental pulp constructs[16]. The cells were counted using pathohistometric analysis, the numbers of cells per microscope field with 5 random microscope fields being counted per specimen using a light microscope (Vista vision, VWR Scientific, West Chester, PA) at × 200 magnifications with a reticule[18]. Construct degradation was measured as an area of scaffold that no longer existed using a light microscope at × 200 magnifications with a reticule.

Assessment of mineralization capacity of SHED

SHED and L929s were cultured and treated with LILIP using the same energy density and 6, 12, 18, and 36 s of irradiation time which was described previously. The SHED and L929s cells were incubated with DMEM mineralization induction media, supplemented with 10 mmol/L sodium β-glycerophosphate (Sigma, St. Louis, MO, United States), for 28 d. The mineralization was assessed by a von Kossa staining (Diagnostic BioSystems, Pleasanton, CA)[19]. The mineralized cultures were fixed with 10% buffered formalin for 30 min. Subsequently, they were washed and stained with von Kossa silver and exposed to ultraviolet light for 30 min. Then cells were treated with 5% sodium thiosulfate for 2 min and washed again. The mineralization capacity of each cell line was determined and compared by measuring the density of mineral nodules formed in each cell type using the tagged image format (tif) image for manipulation in Adobe Photoshop (Adobe Systems, San Jose, CA). Mineralization was measured in three random areas of each specimen.

Statistical analysis

The data were analyzed using an analysis of variance statistical test, followed by Scheffe's multiple comparison tests between treatment groups (Statview, SAS Institute Inc., Cary, NC). A P value of $P < 0.05$ was considered statistically significant.

RESULTS

LILIP output power and energy density

The output power setting of 20 mW of the GaAlAs laser was measured by the laser energy meter as 16.83 mW reaching the SHED through the plastic base of the 6 well plates. After applying the LILIP energy density for 6, 12, 18, and 36 s, the energy density was calculated using a formula to be 0.4, 0.8, 1.2, and 2.4 J/cm^2.

Nutritionally deficient FBS concentration for SHED

Prior to experimentation a pilot study of the effects FBS concentrations (10%-1%) within the DMEM culture media found that a 2.5% FBS concentration, was the minimal concentration of FBS necessary to avoid SHED death and reduced cell proliferation. The 2.5% FBS concentration met the criteria[12,13,15] to be the conditions of SHED nutritional deficit, and the 10% FBS concentration was the optimal nutritional condition.

SHED proliferation following LILIP

A pilot study revealed that the maximum SHED responses were seen 8 d or more following exposure to LILIP, consequently the SHED responses in this present study were measured 8 d after exposure to LILIP. SHED proliferation within the dental pulp constructs varied after exposure to the 0.4, 0.8, 1.2, and 2.4 J/cm^2 LILIP energy densities ($P < 0.05$). The maximum proliferation of SHED in nutrient deficient FBS media was 218% after exposure to a 1.2 J/cm^2 LILIP energy density. SHED grown in nutritional deficient media after exposure to a 0.4, 0.8, and 1.2 J/cm^2 LILIP energy density, proliferated by 167%-218% compared to the untreated (non-LILIP) control group ($P < 0.05$). SHED exposed to a 0.4, 0.8, and 1.2 J/cm^2 LILIP energy density, and grown in optimal nutritional conditions and proliferated by 147%-164% compared to the untreated (non-LILIP) control group ($P < 0.05$). The exposure of SHED to the highest LILIP energy density (2.4 J/cm^2) caused a reduction of the cell proliferation of up to 73% of the untreated (non-LILIP) control ($P < 0.05$). The nutrient deficient (2.5%) FBS culture media and optimal (10%) FBS culture media had little effect on the loss of SHED proliferation following exposure to the highest (2.4 J/cm^2) LILIP energy density ($P > 0.05$). The loss of proliferation (62%)

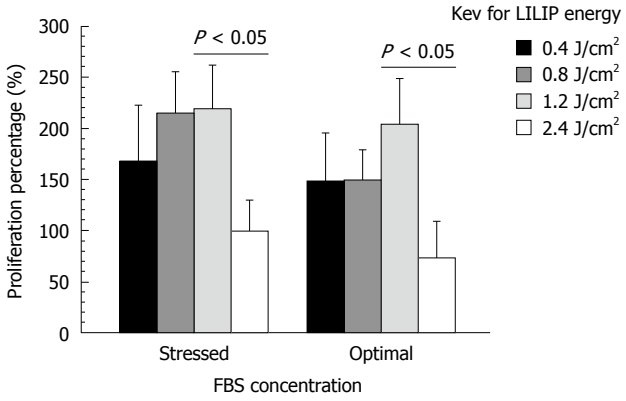

Figure 1 Bar chart of the stem cells from human exfoliated deciduous teeth proliferation within the dental pulp constructs with different low intensity laser irradiation phototherapy energy densities and different fetal bovine serum concentrations. LILIP: Low intensity laser irradiation phototherapy; FBS: Fetal bovine serum.

of L929 was less than the loss of proliferation (73%) of SHED after exposure to the highest (2.4 J/cm^2) LILIP energy density (Figure 1).

SHED mineralization following LILIP

The amount of mineral produced by SHED varied 28 d after exposure to 0.4, 0.8, 1.2, and 2.4 J/cm^2 LILIP energy densities ($P < 0.05$). SHED produced (106%-255%) more mineral than L929 cells ($P < 0.05$). The amount of mineral produced by SHED increased over time up to 28 d ($P < 0.05$). The 0.8 and 1.2 J/cm^2 LILIP energy densities were the most effective at stimulating the SHED to produce minerals over 28 d ($P < 0.05$) (Figure 2). The 0.8 and 1.2 J/cm^2 LILIP energy densities increased the mineralization of the SHED from 150%-700% compared to untreated (non-LILIP) control SHED.

SHED degradation of constructs following LILIP

The degradation of dental pulp constructs was affected by LILIP ($P < 0.05$). The dental pulp constructs grown in optimal nutritional conditions exposed to a 0.8 J/cm^2 or 1.2 J/cm^2 LILIP energy density had 13% to 16% more degradation than the untreated (non-LILIP) control groups ($P < 0.05$). The other LILIP energy densities were less effective at causing the degradation of dental pulp constructs (Figure 3).

DISCUSSION

Regenerative endodontic procedures are successful because SHED and other dental stem cells can regenerate dentin and dental-pulp tissues[20]. The stimulation of SHED to increase proliferation, mineralization and scaffold degradation can be important to ensure that endodontic healing is quick and effective. This is the first investigation of using LILIP to control SHED proliferation, mineralization and construct degradation. This is also the first investigation to determine the optimal and injurious ranges of LILIP energy densities to enhance and inhibit the activity of a dental stem cell line.

The GaAlAs laser was used with an output power setting of 20 mW, but the laser energy meter measured only 16.83 mW reaching the SHED through the plastic base of the 96 well plates. The loss of 15.9% of the laser energy was factored into the energy densities (0.4, 0.8, 1.2, and 2.4 J/cm^2) of this present study. A limiting factor in some previous laser studies[15,21-23] is that the energy densities reaching the cells were not measured by a meter, and so it is not clear what actual energy density was used. In this study, the LILIP parameters (wavelength, power output, irradiated area), were kept constant, except the irradiation times (6, 12, 18, and 36 s, and their corresponding energy densities (0.4, 0.8, 1.2, and 2.4 J/cm^2). The GaAlAs laser wavelength was 830 nm, which is ideal for LILIP[22,23], but low energy compared to other laser types.

The effect of LILIP has been studied on several cell types[12-14]. A previous study found that LILIP can increase the proliferation of DPSCs, by measuring their cell mitochondrial activity using the MTT assay[15]. However, it is not clear what the precise change in the rate of DPSCs proliferation was, or if the 20 mW and 6 s of LILIP energy density[15] was the most optimal, since no other energy densities were investigated, or if DPSCs and SHED used in this present study, share similar responses to LILIP.

The present study discovered that the LILIP energy density could enhance or reduce SHED proliferation and degradation within dental pulp constructs. SHED proliferation increased following exposure to 0.4, 0.8, and 1.2 J/cm^2 LILIP energy densities following culture in both the optimum and nutrient deficient FBS conditions. After prolonged exposure to 2.4 J/cm^2 LILIP energy density, the proliferation of SHED was inhibited. The results indicate that a 1.2 J/cm^2 LILIP energy density is optimal to enhance SHED proliferation. This is consistent with previous research demonstrating that LILIP can stimulate cell proliferation in a narrow energy range, and with low energy density[24]. Excessive LILIP energy densities can inhibit cell proliferation[12,23]. The range of energy densities in this current study which could enhance or reduce SHED activity is in accordance with the Arndt-Shultz Law[25]. The Arndt-Shultz Law predicts that a small amount of laser or other source of energy will increase physiological activity, and that a larger amount of the energy will kill cells[12,23]. In this study, the energy stimulation range was between 0.4-1.2 J/cm^2 and the energy inhibition occurred at a 2.4 J/cm^2 energy density.

SHED are beneficial for regenerative endodontics because they can differentiate into mineralizing cells which can regenerate teeth[26]. In the von Kossa staining part of the present study, the SHED treated with mineralization induction media, we observed to deposit substantial amounts of mineral nodules (black color). The formation of mineral nodules suggests that the SHED differentiated into an odontoblast-like type of cell[27]. The SHED had a greater capacity for mineralization than the control L929 cultures. All the cell cultures showed ascending

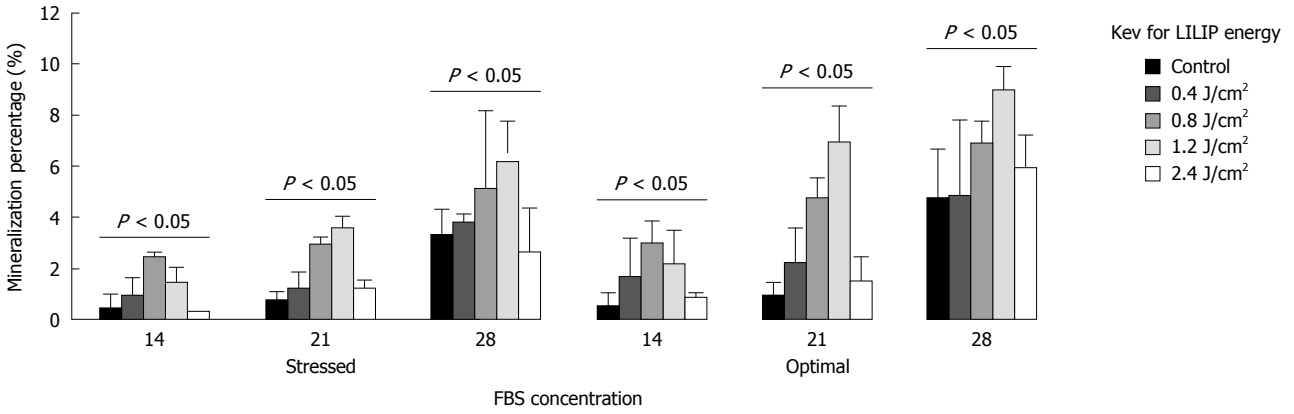

Figure 2 Bar chart of the mineralization percentages of stem cells from human exfoliated deciduous teeth cultures with different low intensity laser irradiation phototherapy energy densities and different fetal bovine serum concentrations. LILIP: Low intensity laser irradiation phototherapy; FBS: Fetal bovine serum.

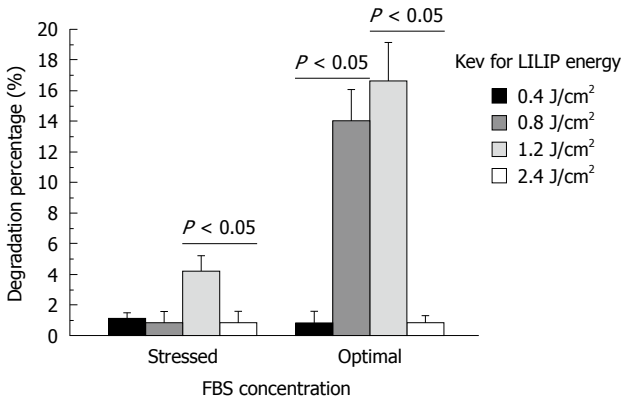

Figure 3 Bar chart of the degradation within the dental pulp constructs with different low intensity laser irradiation phototherapy energy densities and different fetal bovine serum concentrations. LILIP: Low intensity laser irradiation phototherapy; FBS: Fetal bovine serum.

mineralization percentages from 14-28 d regardless the cell line or the FBS concentrations. The cultures supplemented with optimal FBS concentration showed higher percentages of mineralization than the stressed cultures. This indicates that nutrient deficient SHED has a lower capacity for mineralization, suggesting that a nutrient deficit can reduce cell mineralization activity.

The molecular mechanism whereby LILIP can increase cell activity is reported to be its ability to increase the concentration of calcium in the cytoplasm from the mitochondria[9,28,29]. Consequently, the calcium transported into the cytoplasm can increase the rate of cell mitosis and improve cell proliferation. Further research is needed to identify how the molecular mechanisms of cells can be targeted to cause them to proliferate, differentiate and mineralize, as an alternative to the traditional use of growth factors for this purpose[4].

In conclusion, a 1.2 J/cm^2 energy density of LILIP enhances SHED proliferation, dental pulp construct degradation, and mineralization. These results are significant because SHED and other dental cell proliferation, dental pulp construct degradation, and mineralization are needed to make regenerative endodontics quick and effective. Future clinical research is needed to more completely identify the regeneration benefits of using LILIP, such as following the accidental exposure of the dental pulp, Cvek pulpotomy, tooth revascularization and regeneration.

COMMENTS

Background

This is the first article to describe using low intensity laser irradiation phototherapy (LILIP) to control stem cells from human exfoliated deciduous teeth (SHED) proliferation, mineralization and construct degradation. This is also the first article to determine the optimal and injurious ranges of LILIP energy densities to enhance and inhibit the activity of a dental stem cell line.

Research frontiers

A hotspot of dental research is to develop new therapies which can promote dental tissue healing and regeneration. LILIP could be used to activate SHED and potentially other stem cells to regenerate missing dental tissues.

Innovations and breakthroughs

At the current time, lasers are most often used to cut soft dental tissues. LILIP is a new type of laser therapy. LILIP could be used more frequently in future dental practice to regenerate missing tissues for patients.

Applications

Future clinical research is needed to more completely identify the applications of using LILIP, such as to promote the healing of the exposed dental pulp and in conjunction with Cvek pulpotomy, tooth revascularization and regeneration procedures.

Peer review

The reviewers found the article to be innovative and interesting. The article is significant because it is the first article to evaluate the effects of LILIP on SHED within dental pulp constructs.

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BRIEF ARTICLE

Ozone action on *Streptococcus mutans* and *Lactobacillus fermentum*: A pilot study

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Abstract

AIM: To study the effectiveness of ozone in the elimination of cariogenic bacteria, followed with fluoride supplements.

METHODS: Sixty extracted teeth free of caries were used, and five groups were constituted. In Group Ⅰ, the teeth were immersed in artificial saliva. In Group Ⅱ, the teeth were inoculated with *Streptococcus mutans* (*S. mutans*) and immersed in artificial saliva. In Group Ⅲ the teeth were inoculated with *Lactobaccilus fermentum* (*L. fermentum*) and immersed in artificial saliva. In Group Ⅳ the teeth were inoculated with *S. mutans* and *L. fermentum* and immersed in artificial saliva and the teeth in Group Ⅴ were inoculated with *S. mutans* and *L. fermentum*, and were subjected to the application of ozone and to the action of a fluoride mineralizing gel. DIAGNOdent was used to evaluate the caries of the teeth 3 wk after inoculation of bacteria and after that the teeth of Group Ⅴ were subjected to the application of ozone during 60 s, by HealOzone. After the application of ozone, products of the remineralization kit supplied by the manufacturer were applied daily, during 30 d. At the end samples were collected for analysis and evaluation of bacterial activity by polymerase chain reaction.

RESULTS: Regarding the value of caries, obtained *via* DIAGNOdent, in the initial measurement the groups are homogeneous (P = 0.730). There was an increase in DIAGNOdent values, presenting statistical significant difference regarding the initial measurement in all groups (P < 0.001), except in group Ⅰ - only artificial saliva - which shows that the artificial carie model was effective. Comparing the initial and final measurements for each of the 60 teeth, it can be observed that in 9 teeth (15.0%) there was a decrease in values between the two measurements, one (1.7%) retained the same values in the two measurements and in the remaining 50 cases (83.3%) there was increase in values between the initial and final measurements. It should also be noted that in the teeth inoculated with *S. mutans* + *L. fermentum*, there was an increase of the values in 100% of cases, and in all groups except the group with artificial saliva, there is a more frequent increase in the values. In group Ⅴ, subject to the application of ozone, bacterial DNA was not detected, in group Ⅳ, bacterial DNA was detected.

CONCLUSION: Ozone was effective in the elimination of the study bacteria.

Key words: Dental caries; Cariogenic agents; *Lactobacilus fermentum*; *Streptococcus mutans*; Ozone

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INTRODUCTION

Currently dental caries can be defined as a multifactorial and infectious disease, characterized by a localized hard tissue demineralization of tooth, resulting from acidic products, through bacterial fermentation of carbohydrates[1,2].

Clarke[3] (1924) identified *Streptococcus mutans* (*S. mutans*) in carious lesions and 36 years later Fitzgerald and Keyes found that these bacteria were capable of inducing caries in hamsters. A number of studies in humans have shown that caries are a confirmation of bacterial infection, primarily mediated by *S. mutans*, which is transmited through the saliva within the family unit. *Lactobacillus fermentum* (*L. fermentum*) is also associated with the caries process, but is not responsible for it, since it does not have the capacity for the adherence like *S.* mutans[3].

Until today no single factor was found as a caries inducer, instead several factors are proposed such as: microorganisms, diet, teeth and saliva, resulting in the metabolic activity of bacteria that will ferment diet carbohydrates to produce lactic acid[1,2,4]. Metabolic activity leads to changes of pH on the interface of the tooth surface and bacterial deposits, producing an imbalance between the enamel and the fluid of the plaque, causing the loss of tooth mineral when pH decreases, or the mineral gain when the pH increases[1]. The cumulative result of these processes can be demineralization or remineralization, with mineral loss, leading to dissolution of the dental tissues and the formation of caries[1].

In strategies for management of caries, emphasis has been given to preventive treatments and remineralizing procedures in the early detected stages[1,5]. In the presence of caries we have various treatment options. The decision in cavitated teeth becomes simpler, since the treatment choice is conventional drilling and filling[5]. In the lesions that are not cavitated, the decisions become more complex, two types of approach can be selected: invasive, assuming that the lesion is active and will progress; or a conservative approach, in order to stop the progression of caries. The latter approach is where emphasis has been placed, leading to more research in this area involving fluoride products and the remineralization process[6]. These approaches can be divided into selective invasive interventions and non-invasive interventions, in which ozone is included[7]. The non-invasive interventions focus on caries prevention and the preservation of demineralized enamel and dentin, but without cavitation. Under these conditions "restitutio ad integrum" is thought to be possible, by removing bacteria and products of their metabolism and allowing the remineralizing process using fluoride[7].

The antimicrobial effects of ozone have been known for many years[5]. The direct application of this gas on coronary or root surfaces seems to have a sterilization effect[8]. It is mentioned that it is able to slow, stop, or reverse the carious process[7]. It is believed that the ozone is also useful in reducing the microbial flora in cavitated lesions, before proceeding to filling[7]. Although ozone is not a radical, it is the third most potent oxidant[9]. Due to its oxidative power, it induces the destruction of the cell walls and membranes, and cytoplasm of bacteria, fungi and certain viruses[10,11]. The use of ozone in dentistry has been advocated for the sterilization of cavities, root canal, periodontal pocket and herpetic lesions[9]. However, its major indication is in the treatment of caries[4,5]. The ozone procedure involves the application of ozone gas, the use of remineralizing agents and an oral hygiene kit to give to the patient, in order to promote the process of remineralization.

The present work aims to assess the effectiveness of ozone in the elimination of cariogenic bacteria.

MATERIALS AND METHODS

Teeth preparation

Sixty extracted premolars and molars without caries were selected and stored in a saline solution. The extracted teeth were set in acrylic blocks and numbered. Before using the DIAGNOdent® pen 2190 (KAVO GmbH, Biberach, Germany) to evaluate the caries, the teeth were cleaned, dried and selected according the value obtained with DIAGNOdent. An occlusal recess of small size (0.08 mm wide, 1 mm deep and 3 mm in length) was carried out, with a 008 cylindrical diamond drill, placed in turbine with cooling air/water.

Artificial saliva

Artificial saliva solution was used and prepared based on a modification of the basal medium mucin (BMM) medium with modified Shellis artificial saliva, whose composition is described in the Table 1. The bacteria used were *S. mutans* (ATCC reference: 25 175) and *L. fermentum* (ATCC reference: 14 932). These bacteria were grown and maintained according to the instructions in the ATCC (American Type, Culture and Collection). For inoculation of the samples, bacteria were prepared in phosphate buffered solution (PBS) suspensions. This was subsequently diluted to the final value of 10^5 colony forming units for each bacteria of saliva used for this study. Artificial saliva was supplemented with 2% glucose (20 g/L).

Constitution of groups

Five groups were formed: Group Ⅰ - 10 teeth, immersed in artificial saliva; Group Ⅱ - 10 teeth, inoculated with *L. fermentum* with a bacterial load of 10^5 and immersed in artificial saliva; Group Ⅲ - 10 teeth, inoculated with *S. mutans* with a bacterial load of 10^5 and immersed in artificial saliva; Group Ⅳ- 10 teeth, inoculated with *L. fermentum* and *S. mutans* with a total bacterial load of 2×10^5 and immersed in artificial saliva; Group Ⅴ - 20 teeth inoculated with *L. fermentum* and *S. mutans* with a total bacterial load

Table 1 Composition of artificial saliva, modification of basal medium mucin Medium with modified artificial Shellis saliva

	g/L
Yeast extract	5
Proteose peptone	10
Potassium chloride (KCl; 74.5 g/mol)	1.2
Sodium hydrogen carbonate (NaHCO3; 84 g/L)	0.6
Potassium thiocyanate (KSCN; 97.18 g/mol)	0.23
Sodium Di-hydrogen phosphate (Na_2HPO 4.12 H_2O; 358 g/mol)	0.9

of 2×10^5, subject to the application of ozone followed by fluoride remineralizing gel (supplied by the manufacturer of HealOzone, with sodium fluoride 0, 24% w/w) and immersed in artificial saliva.

All teeth during the experimental protocol were kept in an incubator at 37 ℃ with permanent stirring at 150 r/min. All procedures performed during the experimental protocol were performed in a laminar flow chamber. The teeth were immersed in artificial saliva and inoculated in accordance with the specifications for each experimental group. DIAGNOdent was used to evaluate the teeth 3 wk after inoculation, as well as the pH, using a pH measuring tape (pH 1-10 Universalindikator Merck).

Ozone application

The application of Ozone was made with HealOzone® in group V (KAVO GmbH, Biberach, Germany) 3 wk after bacterial inoculation. Before ozone application, the teeth were removed from the medium and washed with a sterile saline solution, and ozone was applied for 60 s. For ozone application a silicone dome was used, which was selected according to the size of each tooth, and changed between each tooth. After application of the ozone a reducing solution was applied, supplied by the manufacturer, for 60 s. Then the teeth were immersed in fresh medium again. Glucose at 0.05% was added to the new medium, also composed of artificial saliva. The bottles were flushed with sterile saline solution before the new medium was inserted. 30 d later, the teeth were brushed with a tooth brush and tooth paste provided by the manufacturer of HealOzone. At the end of brushing a fluoride spray was applied, also supplied by the manufacturer, as a remineralization protocol once a day.

Sample preparation

Seven weeks after inoculation, a collection of Groups Ⅳ and V was made, prepared by excavation of cavities, with a dentin sterile excavator. The samples were stored in PBS and 25% glycerol. The extraction of total DNA from bacteria was performed by the method of proteinase K. Extraction was performed using the protocol of bacteria Lysis Buffer from Roche. After the extraction, the DNA was quantified.

Statistical analysis

Statistical analysis of results shows the mean values and standard deviation. Comparison between groups was performed using Kruskal-Wallis test with multiple comparisons and adjusted in each group, by the Wilcoxon test. Comparisons between the initial and final measurements in each tooth and their relation with group was performed using a χ^2 test with simulation by the Monte Carlo method ($P < 0.001$). All data analysis was performed by SPSS, version 19, at a significance level of 5%.

RESULTS

Regarding the value of caries, obtained with DIAGNOdent, in the initial measurement groups are homogeneous ($P = 0.730$) but the second measuring statistical difference exist between groups, the differences being detected between groups: group I and group V ($P < 0.001$), group I and group Ⅳ ($P = 0.014$), group Ⅳ and group V ($P = 0.036$).

There was an increase in DIAGNOdent values, presenting statistical significant difference regarding the initial measurement in all groups, except in the group I , only artificial saliva, see Table 2.

Comparing the initial and final measurements for each of the 60 teeth, it can be observed that in 9 teeth (15.0%) there was a decrease in values between the two measurements, one (1.7%) retained the same values in the two measurements and in the remaining 50 cases (83.3%) there was an increase in values between the initial and final measurements. Although changes are associated with the group, a decrease of the values can be expected only in the group which used artificial saliva and an increase in teeth with *S. mutans* or *S. mutans* + *L. fermentum*. The maintenance of values between measurements occurred in the *L. fermentum* group. It should also be noted that in the teeth inoculated with *S. mutans* + *L. fermentum* there was an increase of the values in 100% of cases, and in all groups except the group with artificial saliva there is a more frequent increase in the values (Table 3).

In group V, subject to the application of ozone, bacterial DNA was not detected. In group Ⅳ, bacterial DNA was detected, polymerase chain reaction amplification curves, for some teeth of group Ⅳ are presented in Figure 1.

DISCUSSION

At the beginning of the experimental work, all teeth were free from caries. The highest values accepted, quantified by DIAGNOdent® pen 2190, were 12. According to the recommendations of KaVo, this corresponds to the threshold for considering the tooth tissue normal and healthy. The diagnosis of caries lesions is made after visual and tactile examination of tooth surfaces, however it may be necessary the use auxiliary diagnostic tests[1]. A tool developed for the diagnosis and quantification of caries is DIAGNOdent, which is based on tissue fluorescence induced by laser light. In interpreting the values obtained, it is necessary to take into consideration that there may be false positives. These can occur in the fol-

Table 2 Results of DIAGNOdent, expressed as mean ± SE, quartiles and range of variation

Material	Med	*n*	Mean	Structural equation modeling	Min	Max	P25	P50	P75	Med1 *vs* Med2
Artificial Saliva	1	10	6.10	0.795	2	11	4.75	6.00	7.50	0.759
	2	10	8.20	4.54	0	45	0.00	1.00	14.75	
L. fermentum	1	10	5.40	0.792	2	10	3.00	5.50	7.25	0.011
	2	10	19.40	6.31	2	63	7.00	9.50	27.50	
S. mutans	1	10	5.60	0.859	2	9	2.00	6.50	8.00	0.011
	2	10	30.00	10.33	0	99	9.25	15.50	47.00	
S. mutans + *L. fermentum*	1	10	6.00	0.978	2	12	4.50	5.00	8.50	0.005
	2	10	31.90	3.29	16	47	25.00	29.50	43.75	
S. mutans + *L. fermentum* ozone + remin	1	20	5.15	0.782	1	12	2.25	4.00	8.75	< 0.001
	2	20	45.95	5.90	20	99	24.50	37.00	68.50	
Comparisons between 5 groups	1	$P = 0.730$								
	2	$P < 0.001$								

S. mutans: *Streptococcus mutans*; *L. fermentum*: *Lactobacillus fermentum*.

Table 3 Measurements difference for each of 60 teeth

			Group					Total
			Artificial saliva	*L. fermentum*	*S. mutans*	*S. mutans* + *L. fermentum*	*S. mutans* + *L. fermentum* + ozone	
Difference	Decreased	*n* (%)	7	1	1	0	0	9
		Residue	5.5	-0.5	-0.5	-1.5	-3.0	
	Maintenance	*n* (%)	0	1	0	0	0	1
		Residue	-0.2	0.8	-0.2	-0.2	-0.3	
	Increased	*n* (%)	3	8	9	10	20	50
		Residue	-5.3	-0.3	0.7	1.7	3.3	
Total			10	10	10	10	20	60

S. mutans: *Streptococcus mutans*; *L. fermentum*: *Lactobacillus fermentum*.

lowing situations: teeth with plaque; fillings with fluorescent composites; proximity of the pulp; food waste; prophylactic pastes; remineralized cavities; increased natural fluorescence and patients exposed to radiation.

Three weeks after bacterial inoculation, the presence of caries was evaluated in all groups, using DIAGNOdent® pen 2190. Some authors claim that the diagnostic technologies such ECM (Electric Caries Meter) and DIAGNOdent, perform better in the early detection of caries in occlusal surfaces, compared with visual examination[12]. Goel *et al*[13] in 2009 conducted an *in vivo* study that compares the efficacy of DIAGNOdent with conventional methods (visual, tactile and bitewings radiographs). The authors concluded that DIAGNOdent showed high precision compared to conventional methods for the detection of enamel caries. However, when the cavities reach dentine, although precision is high, it is similar to other diagnostic methods. The DIAGNOdent is the diagnostic method chosen for this work since it allows the quantification of dental caries, has good accuracy, reproducibility, sensitivity and validity[12,13].

Plaque in humans is a complex biofilm comprising hundreds of microbiological species[14]. The culture plaque using the BMM medium, exhibits similar growth rates behaviour to the natural. Oral fluids are the major source of nutrients to bacterial plaque[14]. Several studies simulating these oral fluids *in vitro* contained as main components mucin, yeast extract and/or peptones. One of these formulations is the BMM medium, which has been widely used for *in vitro* studies of oral bacteria[14]. The presence of yeast extract and peptones contributes to the presence of a variable concentration of peptides, vitamins and ions in the medium[14]. Shellis in 1978 developed an artificial saliva, chemically defined, containing various ions, amino acids, vitamins, growth factors and bovine origin mucin[14]. This has as its major components potassium chloride, sodium chloride, sodium hydrogencarbonate and potassium thiocyanate[14].

The artificial saliva used in this experimental study consisted of a modification of BMM medium, with modified Shellis saliva, which since the system used was a simple one, only comprises two bacteria. To allow a greater bacterial growth prior to inoculation of bacteria on teeth, the artificial saliva was supplemented with 2% glucose, to allowing the formation of lactic acid by fermentation.

After the appearance of the tooth caries and application of ozone, the teeth were immersed in new medium, the same as used earlier, but without glucose supplementation, since the carious lesion was established and the concentration of glucose in yeast extract must correspond to 0.05% and the concentration of glucose in saliva in an adult is 0.01%[15].

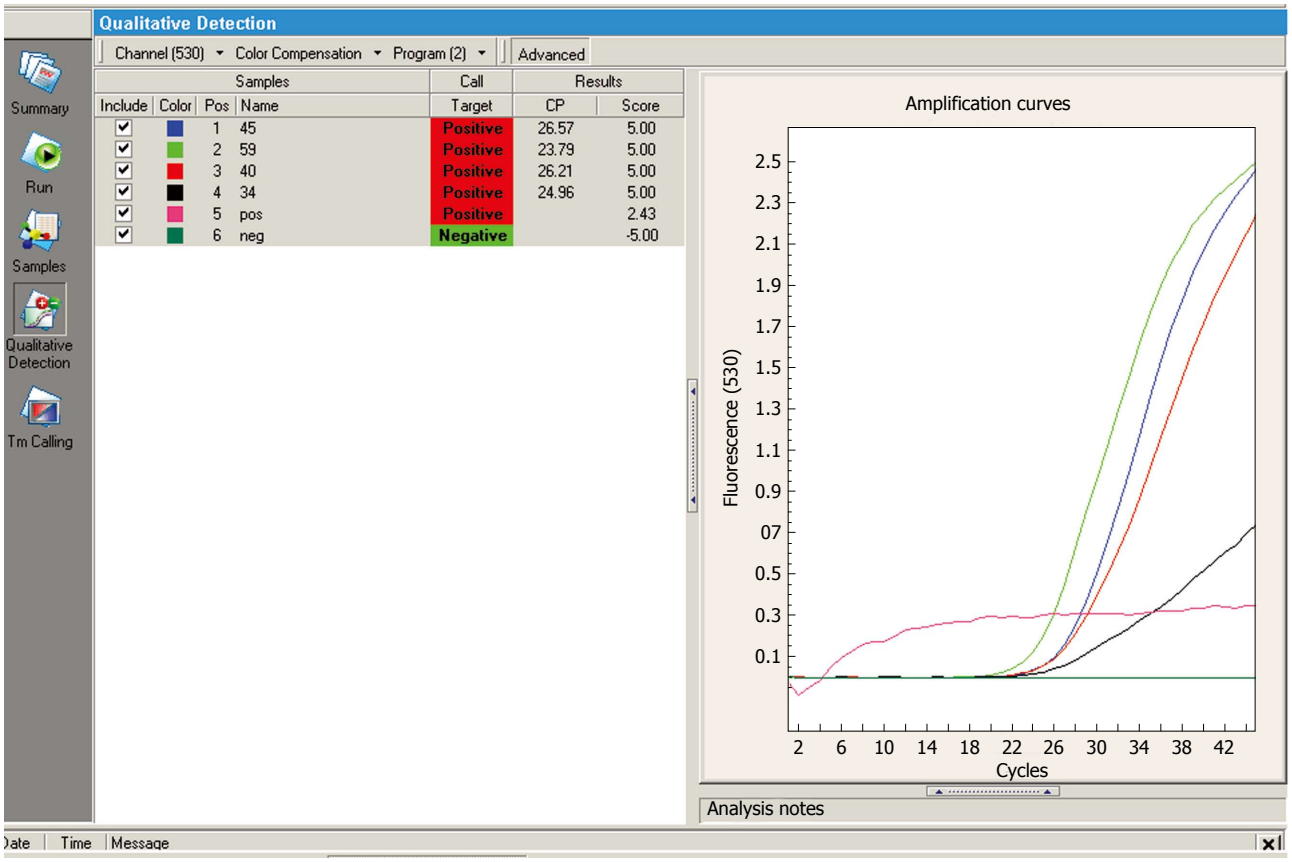

Figure 1 Polymerase chain reaction amplification curves of real-time for the group Ⅳ, inoculated with *Streptococcus mutans* and *Lactobacillus fermentum*.

In order to promote bacterial retention on site and to allow faster development of the caries process, we performed a cavity of small dimensions on the occlusal surface as the occlusal surfaces are particularly susceptible to caries[1].

The antimicrobial effect of ozone has been shown by several studies[11,16]. However there are studies that show conflicting results, such as those done by Baysan *et al*[17] in 2007, which demonstrated that the application of ozone in carious lesions of dentin did not significantly reduce the number of bacteria detected in infected dentin in non-cavitated caries. Castillo *et al*[11] in 2008 conducted an *in vitro* study, which used 41 strains of *S. mutans* including native strains obtained from the saliva of 27 children, which after being cultured, were placed in eppendorf. The ozone was applied to the eppendorf and the results show that after application of ozone for 40 s, no bacteria were viable. Polydorou *et al*[16] in 2006, compared the antibacterial effect of ozone with two dentin adhesive systems. The adhesive systems showed significant antibacterial activity. However, application of ozone has proved a promising in eliminating residual microorganisms in deep cavities and potentially increases the clinical success of fillings. Knight *et al*[18] in 2008 conducted an *in vitro* study to determine the effects of ozone application in dentin before the formation of a biofilm. The study showed that the ozone application prevents the formation of a biofilm of *S. mutans* and Lactobacillus acidophilus for a period of 4 wk.

Studies conducted to evaluate the efficacy of ozone in carious lesions in pits and fissures and roots already have a good level of scientific evidence, since they are randomized. However, there are authors who raise questions about the evaluation method, which is in most cases performed by DIAGNOdent, and as to whether the participants were aware of the treatment administered to them (only the study by Holmes is double-blind) and that many of the studies were conducted by the team members from the main precursor of this therapy, Edward Lynch[10]. Systematic reviews on the subject are unanimous about the fact that more scientific evidence is needed that this therapy can be accepted as an alternative therapeutic approach to early detected caries[5,10].

From the study it can be concluded that the caries induction protocol employed in this study was effective in the development of caries lesions. The application of ozone through HealOzone for 60 s, at a concentration of 2100 ppm was effective in eliminating caries bacteria. No bacterial DNA was detected after the application of ozone, followed by the daily application of the remineralizing products for 30 d. The treatment with ozone is advantageous in minimally invasive dentistry, it maintains the healthy tissue, and does not require anaesthesia, and with a simple and non-time-consuming process presents satisfactory results. More evidence is still needed before the cost-benefit ratio can be assessed.

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COMMENTS

Background

Carious lesions occur due to acid dissolution of the enamel and/or dentin as a result of the metabolism of specific microorganisms, the main ones being *Streptococcus mutans* (*S. mutans*) and *Lactobacillus fermentum* (*L. fermentum*). Ozone is a powerful oxidant that has the ability to eliminate 99% of bacteria, fungi and viruses. Once the bacteria is eliminated, the remineralization may occurs in the treated area.

Research frontiers

The bacterias *S. mutans* and *L. fermentum* are effective in producing tooth decay immersed in artificial saliva, and ozone was able to eliminate them. In this study the authors shows the capability of ozone in their elimination.

Innovations and breakthroughs

This is the first study to demonstrated the capability of bacteria elimination by the ozone, by polymerase chain reaction. Demonstrating that the ozone is highly effective. This study by demonstrated the bacteria elimination, shows that the ozone provides conditions for the remineralization.

Applications

Ozone therapy is advantageous in minimally invasive dentistry, because it maintains healthy tissue, does not require anesthesia and with a simple and non-time-consuming procedure gives satisfactory results.

Terminology

The carie occurs due to acid dissolution of the enamel and/or dentin as a result of the metabolism of specific microorganisms, the main are *S. mutans* and *L. fermentum*. Ozone is oxidant that has the ability to eliminate bacteria, fungi and viruses.

Peer review

The manuscript is good to add some information on the antibacterial effect of ozone in dental caries.

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BRIEF ARTICLE

MMP-8 analysis in gingival crevicular fluid using ELISA and novel chair-side test

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Abstract

AIM: To validate accuracy of a novel chair-side test for matrix metalloproteinase (MMP)-8 as compared to enzyme-linked immunosorbent assay (ELISA) in Periodontal health and disease.

METHODS: Gingival crevicular fluid was collected from 150 subjects, Group 1 (healthy) - 50 subjects, Group 2 (gingivitis) - 50 subjects and Group 3 (chronic periodontitis) - 50 subjects. A chair-side test strip was indigenously prepared using polyclonal antibodies (principle of immunochromatography) to detect the MMP-8 levels. The detection accuracy (sensitivity and specificity) of the MMP-8 levels by chair-side test kit were compared with ELISA at baseline and 3 mo after scaling and root planing among the study population.

RESULTS: The novel chair side test detected MMP-8 levels in accordance with ELISA which at baseline were higher in Group 2 and Group 3 as compared to controls ($P < 0.05$), and these enzyme levels decreased after therapy ($P < 0.05$). The chair-side test could differentiate healthy, gingivitis and periodontitis. The detection accuracy of the chair-side test strip were on par with ELISA (sensitivity 92.9% and specificity of 100%) which were statistically significant ($P < 0.05$). A desire to arouse interest about periodontal health and maintenance in the Indian population provided a strong rationale for us to develop our chair-side test strips to suit our economy. Moreover, this was the first ever effort to develop and validate a chair-side test strip to detect MMP-8 levels in the Indian population. This test can be used on a large scale in private dental practice for the early detection of disease, tapping the sites at risk for disease, alongside helps in patient education and motivation for maintenance.

CONCLUSION: This study shows that the novel chair side test kit detects MMP-8 levels a biomarker of periodontal disease progression accurately making it a good chair side diagnostic tool. Further, it is cost effective and time saving which can make it applicable in private dental practice on a large scale for the early detection of periodontal disease.

Key words: Chair-side test; Chronic periodontitis; Gingival crevicular fluid; Matrix metalloproteinase-8; Periodontal health

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INTRODUCTION

Periodontal diseases are chronic inflammatory diseases of

the supporting structures of the teeth. Though periodontitis is often triggered by periodontopathogens, its clinical outcome is highly influenced by the host local immune response[1]. In view of the irreversible nature of progressive periodontitis, an early diagnosis and treatment of this disease is important. An early diagnosis will help prevent further irreversible loss of connective tissue attachment of teeth and adjacent alveolar bone associated with periodontal disease[2,3]. It is a well-established fact that the host immune products in periodontitis are synthesized locally and appear within the gingival crevicular fluid (GCF). This makes GCF ideal for obtaining diagnostic information of periodontal health or disease status[4]. The markers thus identified include cytokines, prostaglandins, bacterial- as well as host-derived enzymes, and connective tissue-degradation products, alongside bone matrix components that are primarily isolated in the GCF[5].

Matrix metalloproteinases (MMPs) are one such Group of enzymes which play a key role in the mediation of tissue destruction in periodontitis. MMP-8, a collagenase synthesized by neutrophils, is the major metalloproteinase implicated in the degradation and remodeling of the extracellular matrix. MMP-8 has a strong affinity towards type I collagen, which is present in abundance in the periodontal tissues[6]. There is growing evidence which indicate that a predominant association exists between increased GCF collagenase activity and disease progression, as it is extensively distributed in diseased periodontal tissues. A significant decrease in the GCF MMP-8 activity has been demonstrated following successful non-surgical periodontal therapy[7,8]. The potentiality of MMP-8 as a biomarker of periodontal disease progression is evident from literature.

Keeping this in view, the first chair-side, point-of-care dip-stick test based on the principle of immunochromatography utilizing monoclonal antibodies was developed and tested successfully to detect MMP 8 levels in GCF[9]. However, it was not popularized to be used on a large scale in private dental practice, probably because there were no further studies reported in literature evaluating and validating its use on a large sample size; Secondly, cost of the test kit could have escalated with the use of monoclonal antibody.

This fact provides a strong clinical rationale to indigenously develop a point-of-care test utilizing polyclonal antibody which is cost effective compared to monoclonal based test kit for MMP-8 detection with a diagnostic accuracy on par with enzyme-linked immunosorbent assay (ELISA). To overcome this limitation we indigenously developed a novel chair side point of care dip stick test based on the principle of immunochromatography utilizing polyclonal antibody (cost effective) instead of monoclonal antibody to detect MMP-8. The objective of the current study is to validate diagnostic accuracy (sensitivity and specificity) of our indigenously developed a novel chair-side test kit compared to that of ELISA in periodontal health and disease. Further to check the cost-effectiveness to suit the economies of developing countries like India.

MATERIALS AND METHODS

Study population

The study population consisted of 187 subjects (77 males and 90 females), 30-39 years of age, who were screened from the outpatient section of Department of Periodontics, Krishnadevaraya College of Dental Sciences and Hospital, Bangalore and 150 subjects (75 males and 75 females) were recruited for the study that was conducted during April to May 2010.

The following criteria prevented the patients from being included in the study Groups: medically compromised patients requiring prophylactic antibiotics, patients on antibiotic therapy within the last 6 mo, patients who had received any form of periodontal therapy surgical or non-surgical within 6 mo of baseline examination, smokers, pregnant patients, patients with recent orthodontic treatment, pulpal or periapical involvement on the qualifying teeth.

Each subject underwent a full mouth periodontal probing and charting, the subjects were categorized into three Groups based on clinical examination of gingival index (GI) (Loe and Silness, 1963), plaque index (PI) (Turesky Gilmore Glickman modification of the Quigley and Hein plaque index, 1970) and probing pocket depth (PPD) using a UNC15 probe. Fifty subjects with clinically healthy periodontium, mean PI $\leqslant$ 1, mean GI $\leqslant$ 1, no PPD, were included in Group 1. Group 2 (gingivitis Group) consisted of Fifty subjects with gingival inflammation as indicated by the mean PI $\geqslant$ 2, GI $\geqslant$ 2 and the absence of PPD. Group 3 (chronic periodontitis) consisted of fifty subjects with a mean PI $\geqslant$ 2, GI $\geqslant$ 2 and PPD $\geqslant$ 5 mm falling in the category of severe periodontitis as per the classification. Therefore of the patients recruited in the study, Group 1 had individuals with a healthy periodontium, Group 2 included individuals with gingivitis and no attachment loss and those in Group 3 were diagnosed to have severe chronic periodontitis. Groups 2 and 3 were treated with non surgical approach, scaling and root planning was done using area-specific Gracey currets (Hu friedy) and ultrasonic scalers.

Subjects satisfying the above criteria for enrolment were selected consecutively, and ethical clearance for the study was obtained from the institutional ethical review board, Rajiv Gandhi University of Health Sciences, in accordance with the guidelines of Indian council of Medical research. Written informed consent as per the declaration of Helnski 2008 was obtained from those who agreed to participate in the study.

Site selection and collection of GCF

The study was a triple blind prospective cross-sectional study in which clinical examination, Group allocation and sample site selection was performed by one examiner, the

samples were collected on the subsequent day by the second examiner, and a third examiner carried out the post-treatment clinical examination. This was done to ensure masking of the sampling examiner and to prevent contamination of GCF with blood associated with the probing of inflamed sites. One site per subject was sampled, in gingivitis patients, site with most severe clinical inflammatory signs (in gingivitis cases) or greatest amount of probing depth (in chronic periodontitis cases), along with radiographic confirmation of alveolar bone loss, and the same test site was selected for the after-treatment Group.

On the subsequent day, after drying the area with blast of air, supragingival plaque was removed without touching the marginal gingiva to eliminate the possibility of saliva contamination and thereafter GCF was collected. A standardized volume of 3 μL was collected from each test sites using the calibration of colour coded 1-5 μL calibrated volumetric microcapillary pipettes (Sigma Aldrich, St. Louis, MO) with an extracurricular (unstimulated) method. The test site, which did not express any volume of GCF, and microcapillary pipettes suspected of being contaminated with blood and saliva were excluded from the study. In such cases the sample was obtained from the tooth that showed the next highest PPD in the same patient. The GCF collected was transferred to eppendorf tubes containing 0.5 mL of phosphate buffer saline and stored at -70 ℃ until the time of assay.

Though cumbersome this method of GCF collection was adopted as to prevent protein binding to the paper strips and the risk of sample evaporation.

MMP-8 assay

The samples were assayed for MMP-8 levels using commercially available ELISA kit. The assays were conducted according to the manufacturer's instructions. Highly sensitive ELISA kit (Booster Biological technology Co., LTD, Shanghai) was used to detect the enzyme levels in the sample. This kit reported an assay of sensitivity of < 10 pg/mL. In relation to specificity, the manufacturer reported no significant cross reactivity or interference for the ELISA kit. The samples were run in duplicates. The kit made use of biotinylated anti-human MMP-8 antibody and Avidin-Biotin-Peroxidase Complex. Absorbance of the substrate colour reaction was read on ELISA reader using 450 nm wavelengths. The total MMP-8 level was determined in nanograms (ng), and the calculation of the concentration in each sample was performed by dividing the amount of enzyme by the volume of sample (ng/mL).

Fabrication of point-of-care test sticks for chair-side monitoring

The chair-side test was fabricated based on the sandwich ELISA principle. Nitrocellulose membrane of pore size 0.45 μm was cut into small strips. One end of the strip was treated with methanol to make the membrane hydrophilic. Methanol was washed off using 0.01 mol/L phosphate buffered saline (PBS) buffer. 1 μL of a primary polyclonal antibody to human MMP-8 was added to the hydrophilic end of the nitrocellulose membrane and allowed to dry. GCF sample collected was transferred to an eppendorf tube containing 0.5 mL of 0.01 mol/L PBS buffer. The test strip was immersed into this tube to allow the sample to bind the primary antibody. The strip was removed and washed thoroughly in the PBS buffer to remove the unbound sample. The strip was then dipped in an eppendorf tube containing secondary antibody to human MMP-8 conjugated with a peroxidase system for 10-15 min. The strips were washed in the buffer again and transferred to another eppendorf tube to which a colour developing solution was added. The solution turns blue in colour for samples positive for MMP-8. A colour change within 5 min was recorded as +++ (strongly positive), a change between 5 to 10 min was recorded as ++ (moderately positive) and a change in colour after 15 min was recorded as + (weakly positive). The individual reading the test results was unaware of the ELISA results to eliminate bias and ensure blinding.

Statistical analysis

Analysis of variance was used to compare all variables between groups and a P value of $\leqslant 0.05$ was considered to be statistically significant. SPSS version 13 was used for all the analysis.

RESULTS

Recruitment of subjects for the study started in the first week of April 2010, recruitment ended by the end of the second week of April. 187 subjects (77 males and 80 females), 30-39 years of age, were screened from the outpatient section of Department of Periodontics, Krishnadevaraya College of Dental Sciences and Hospital, Bangalore. Of the 187 individuals screened only one hundred and fifty subjects (75 males and 75 females) were available for analysis. GCF MMP-8 levels measured by enzyme linked immune sorbent assay were able to distinguish sites with periodontitis from those with gingivitis and healthy sites. All samples in each Group tested positive for MMP-8. The highest mean concentration of MMP-8 was obtained in Group 3 (1948.65 ± 916.44 mg/mL), and the lowest mean concentration was obtained in Group 1 (96.90 ± 30.88 mg/mL). The mean MMP-8 concentration for Group 2 (797.94 ± 185.60 mg/mL) was intermediate between the healthy and periodontitis sites. GCF MMP-8 levels > 1 mg/mL especially helped to differentiate the periodontitis from gingivitis and healthy sites, and 1 mg/mL was used as cut-off point in the chair-side test as previously reported (Tables 1 and 2).

A significant improvement in clinical parameters was observed after treatment (Table 3). κ statistics were performed to know the degree of agreement between the test stick and ELISA results. κ value was 0.959, indicating

Table 1 Descriptive data showing comparison of enzyme-linked immunosorbent assay and test stick results

Visit	Group	Test stick results	MMP-8 (mg/L)		Total
			> 1	≤ 1	
Baseline	Group 1	Positive		1	1
		Negative		49	49
		Total		50	50
	Group 2	Positive	4	0	4
		Negative	8	38	46
		Total	12	38	50
	Group 3	Positive	30	2	32
		Negative	5	13	18
		Total	35	15	50
3 mo	Group 1	Positive		1	1
		Negative		49	49
		Total		50	50
	Group 2	Negative		50	50
		Total		50	50
	Group 3	Positive	13	0	13
		Negative	0	37	37
		Total	13	37	50

MMP: Matrix metalloproteinase.

Table 2 Test strip results at baseline and three month follow-up

Visit	Group	Site	Test result of site	≤ 1 mg/L	> 1 mg/L
Baseline	Group 1	50	Positive	1	0
			Negative	49	0
	Group 2	50	Positive	0	4
			Negative	38	8
	Group 3	50	Positive	2	30
			Negative	13	5
3rd mo	Group 1	50	Positive	0	1
			Negative	0	49
	Group 2	50	Positive	0	0
			Negative	0	50
	Group 3	50	Positive	0	13
			Negative	37	0

a good agreement between the two tests at both baseline as well as the third month follow-up.

DISCUSSION

MMP-8 or collagenase-2 is one of the central biomarkers in the breakdown of periodontal connective tissue during the transition from health to disease[7,10-13] besides it has been found to be a potential candidate for use in diagnostic aids[14,15].

A triple blind prospective study was done to gain an insight into the diagnostic accuracy of the MMP-8 chair-side test and check its cost-effectiveness for application on a large scale. A possible role of MMP-8 as a mediator of periodontal inflammation and a comparison of the levels of MMP-8 in GCF among the three study Groups namely Group1, Group 2, and Group 3 and after treatment in Group 2, and Group 3 was assessed using our chair-side point of care test as well as ELISA. Since there

Table 3 Changes in clinical parameters from baseline to three months following scaling and root planning *n* (%)

Visit	Bleeding on Probing	Probing pocket depth		Total
		≥ 5 mm	< 5 mm	
Baseline	Negative	8 (30.8)	8 (33.3)	16 (32.0)
	Positive	18 (69.2)	16 (66.7)	34 (68.0)
	Total	26 (100.0)	24 (100.0)	50 (100.0)
3 mo	Negative		43 (86.0)	43 (86.0)
	Positive		7 (14.0)	7 (14.0)
	Total		50 (100.0)	50 (100.0)

have been reports on age-dependent changes in inflammatory mediators, we selected subjects in the age Group of 30-39 years to control the influence of age on the levels of MMP-8[16]. Further, a single site was selected for sample collection from each participant, which precluded the pooling of samples from multiple sites. Unstimulated samples were collected as an increase in vascular permeability of the blood vessels following gingival stimulation has been reported[17], suggesting that the levels of MMPs in GCF could be influenced by stimulation in sampling.

To the authors' knowledge, this study was the first of its kind to investigate the GCF levels of MMP-8 in the Indian population. Furthermore, this was the first attempt to tailor a chair-side diagnostic test to detect these enzyme levels in Indian population and suit their economic status as well. Though previous studies have successfully designed a chair-side test it had few shortcomings, firstly, the cost of the test strip which was escalated due to the use of monoclonal antibodies. Second, there was no data on post-treatment assessment of MMP-8 levels using the chair-side test which would have been of great help in patient education and motivation, and to identify the site at risk for disease progression.

The main finding of our study was that a strong association exists between the MMP-8 levels and the degree of inflammation as indicated by the changes in clinical parameters and the enzyme levels. The chair-side test strips also showed good agreement in this accord as depicted by the κ values. The specificity and sensitivity of the test strip were found to be good (sensitivity 92.9% and specificity of 100%).

Test strips were fabricated using polyclonal antibodies instead of the monoclonal antibodies previously used, this cut down the cost of the chair-side test almost threefold. Moreover, the specificity and sensitivity remained on par with the previously designed test, this further confirmed that the specificity and sensitivity obtained with either polyclonal or monoclonal antibodies remained almost the same[18] validating our results.

Even though ELISA is a highly sensitive assay and is widely used to detect various biomarkers to aid in diagnosis of various diseases there were certain limitations of ELISA, like technique sensitivity, the time consuming, a delay in providing results to the patients and the cost

involved favoured the fabrication of an easy to use chair-side test strip.

Our chair-side test strip confirmed that it serves as a good diagnostic tool and helps in early detection and maintenance of patients. However, due to the cross-sectional setting of the present study no definite conclusion can be drawn in this regard. To overcome this limitation of the present study, more number of multicenter trials need to be carried out with larger sample sizes. In the near future the chair-side tests could help in the diagnosis of at risk sites in periodontal patients as well as in the early detection and control of periodontal disease in patients at risk due to various systemic and environmental factors. Currently, a randomized controlled trial on a large sample size is being carried out by our organization to tap the at risk population with our new chair-side diagnostic test strip for MMP-8 detection. Apart from this another trial using the chair-side MMP-8 test is being used to detect MMP-8 levels in saliva samples as well to make it less cumbersome and more cost effective.

In short, it can be said that MMP-8 levels reflects the levels of inflammation in the tissues and this can be maintained at low levels with good patient education and regular maintenance. Currently, ELISA is widely used to detect biomarkers of various diseases. But it has some limitation like technique sensitivity and time consuming and cannot be adopted as a chair-side diagnostic aid. The quest to develop a rapid chair-side diagnostic aid with high validity prompted us to fabricate an indigenous chair-side point of care test.

COMMENTS

Background

Periodontitis is a complex, multifactorial disease, whose progression depends on the interplay between periodontopathogens, environmental factors and the host response. Matrix metalloproteinase-8 (MMP-8) is one of the major enzymes of host response to influence the degradation of the periodontal connective tissues. Many studies have proved this role of MMP-8, a chair-side test kit was also fabricated but was not popularized.

Research frontiers

The detection of MMP-8 by various methods has been carried out regularly. However, a point- of-care chair-side test with diagnostic accuracy on par with the regular methods like enzyme-linked immunosorbent assay as well as economic stability was lacking in developing countries like India. In this study, the authors demonstrated the accuracy of an indigenously prepared chair-side test which was suitable for large scale private practice use as well.

Innovations and breakthroughs

This study confirmed that a strong association exists between the MMP-8 levels and the degree of inflammation as indicated by the changes in clinical parameters and the enzyme levels. The chair-side test strips also showed good agreement in this accord as depicted by the κ values. The specificity and sensitivity of the test strip were found to be good (sensitivity 92.9% and specificity of 100%). Therefore it serves as a good diagnostic tool and helps in early detection and maintenance of patients.

Applications

Thus the indigenously fabricated test can be used along chair-side to detect the MMP-8 levels and identify at risk sites and patients thus aiding in an early diagnosis and good maintenance of periodontal patients.

Terminology

MMPs are matrix metalloproteinases, which represent a Group of Zinc dependent enzymes involved in connective tissue degradation. Of these MMP-8 is the major enzyme with an affinity towards collagens.

Peer review

This study is interesting and the results are useful to the readers. The authors concluded that the novel chair side test kit detects MMP-8 levels a biomarker of periodontal disease progression accurately making it a cost effective and time saving diagnostic tool. It really adds new information. It is a prospective well designed research study with interesting results. The paper is well written with appropriate structure. Thus, it could be accepted for publication.

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases.

Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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