Polytechnic University of Marche
PhD Program in Biomedical Sciences XXXIII° Cycle
Dipartimento di Scienze Cliniche Specialistiche ed Odontostomatologiche

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Nanotechnological Applications in Clinical Dentistry

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2017-2021

Preface

In the last decades, nanoscience in medicine and nanotechnology have become particularly important technology areas worldwide, representing the core of the advances seen in modern dentistry. Indeed, nanodentistry today offers to clinicians a real solution, providing instruments and materials capable to simplifying and improving the quality of the procedures, thus obtaining predictable and satisfactory results, even for the patient.

The present dissertation is based on the research studies conducted during my PhD studies at the Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy, from November 2017 to March 2021.

This thesis includes a general background regarding the employment of nanotechnology in clinical dentistry, followed by three sections dedicated to specific research topics on nanotechnological applications in Preventive, Aesthetic and Restorative Dentistry, in which the following eleven original articles will be referred, by using Roman numerals as follows (Paper I – XI):

I. Tosco V, Monterubbianesi R, Sparabombe S, Orilisi G, Putignano A, Orsini G (2020) In Vitro Investigation of the Effect of Different Remineralizing Agents on Human Enamel. Mod App Dent Oral Health 4(3). MADOHC.MS.ID.000189. DOI: 10.32474/MADOHC.2020.04.000189.

II. Orilisi G, Monterubbianesi R, Tosco V, Conti C, Procaccini M, Putignano A, Orsini G (2020) Effect of a Sodium Fluoride-Releasing Rubber Cup on Hydroxyapatite Crystallinity of Human Enamel: FTIR Spectroscopy Analysis. Dent. Res. Oral Health. 03. 10.26502/droh.0018.

III. Sparabombe S, Monterubbianesi R, Tosco V, Orilisi G, Hosein A, Ferrante L, Putignano A, Orsini G (2019) Efficacy of an All-Natural Polyherbal Mouthwash in Patients with Periodontitis: A Single-Blind Randomized Controlled Trial. Front. Physiol. 10:632. doi: 10.3389/fphys.2019.00632

IV. Monterubbianesi R, Sparabombe S, Tosco V, Profili F, Mascitti M, Hosein A, Putignano A, Orsini G (2020) Can Desensitizing Toothpastes Also Have an Effect on Gingival Inflammation? A Double-Blind, Three-Treatment Crossover Clinical Trial. Int. J. Environ. Res. Public Health Dec 1, 17, 8927.

V. Mascitti M, Orsini G, Tosco V, Monterubbianesi R, Balercia A, Putignano A, Procaccini M, Santarelli A (2018) An Overview on Current Non-invasive Diagnostic Devices in Oral Oncology. Front. Physiol. 9:1510. doi: 10.3389/fphys.2018.01510

VI. Orilisi G, Tosco V, Monterubbianesi R, Notarstefano V, Özcan M, Putignano A, Orsini G (2021) ATR-FTIR, EDS and SEM evaluations of enamel structure after treatment with hydrogen peroxide bleaching agents loaded with nano-hydroxyapatite particles. PeerJ 9:e10606 DOI 10.7717/peerj.10606

VII. Tosco V, Vitiello F, Furlani M, Gatto ML, Monterubbianesi R, Giuliani A, Orsini G, Putignano A (2021) Microleakage Analysis of Different Bulk-Filling Techniques for Class II Restorations: μ-CT, SEM and EDS Evaluations. Materials, 14, 31. https://doi.org/10.3390/ma14010031

VIII. Chiodera G, Orsini G, Tosco V, Monterubbianesi R, Manauta J, Devoto W, Putignano A (2021) Essential Lines: a simplified filling and modelling technique for direct posterior composite restorations. Int. J. Esthet. Dent. 2021;16:168–84

IX. Tosco V, Monterubbianesi R, Orilisi G, Procaccini M, Grandini S, Putignano A, Orsini G (2020) Effect of four different finishing and polishing systems on resin composites: roughness surface and gloss retention evaluations. Minerva Stomatol.;69(4):207-214. doi: 10.23736/S0026-4970.19.04310-3. Epub 2019 Oct 15. PMID: 31633320.

X. Monterubbianesi R, Tosco V, Sabbatini S, Orilisi G, Conti C, Özcan M, Orsini G, Putignano A (2020) How Can Different Polishing Timing Influence Methacrylate and Dimethacrylate Bulk Fill Composites? Evaluation of Chemical and Physical Properties. BioMed Res. Int, Article ID 1965818, 8 pages, 2020. https://doi.org/10.1155/2020/1965818

XI. Tosco V, Monterubbianesi R, Orilisi G, Sabbatini S, Conti C, Özcan M, Putignano A, Orsini G (2020) Comparison of two curing protocols during adhesive cementation: can the step luting technique supersede the traditional one? Odontology. doi: 10.1007/s10266-020-00558-0. Epub ahead of print. PMID: 33128650.

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Background

The great aesthetic demand of patients, the simplification of operative protocols and the need for better physical and mechanical properties of the materials have increased the use of technology in all disciplines of dentistry. Indeed, modern dentistry aims to perform minimally invasive procedures, leading to obtain excellent results thanks to the application of new materials with optimal chemico-mechanical characteristics based on nanotechnological implemented features.

Nanotechnology, deriving from the Greek word “νάνος” (nànos), refers to the technology that deals with small structures or materials of small dimensions, or rather one billionth on the American scale (nanometer, nm=10^{-9}). Nowadays, the developments in the arena of nanotechnology is even more present in dentistry, in all fields of oral diseases, diagnosis, prevention and treatment.

Traumatic injuries and various external harmful agents such as bacteria and acids jeopardize tooth integrity. Periodontal disease and carious lesions are mainly responsible for pathologies affecting the dental hard tissues. Their reparative capability is often insufficient to entirely restore the damaged tissues. For this reason, the prevention of tooth decays and the early treatment of lesions and cavities are priority challenges for clinicians. Nanomaterial-based design is able to mimic some of the mechanical and structural properties of native tissues and can definitely promote biointegration. In this light, the advances in the development of nanomaterials for different applications in preventive dentistry and research, including clinical trials, are increasing. For instance, pure inorganic nanomaterials based on synthetic hydroxyapatite (HA) or its derivatives modified with zinc, fluoride, or carbonate elements, and/or organic nanomaterials consisting on casein phosphopeptide (CPP) stabilized with amorphous calcium



phosphate (ACP) are widely used to remineralize enamel structure and prevent dentin hypersensitivity, in the form of dentifrices, mouth rinsing solutions, and remineralizing gels and pastes. Moreover, zinc carbonate HA nanoparticle clusters provide antibacterial properties and reduce initial biofilm adherence, thus potentially decreasing decay lesions formation.

Furthermore, the application of HA nanoparticles has been described even for improving dental aesthetic treatments. In the last decades, patients' demand for aesthetic dental procedures is growing, and, in particular, the dental whitening (commonly named as bleaching) procedures are becoming incredibly popular in dentistry. Despite its attractiveness, there are possible severe collateral effects related with the chemical oxidants' application for tooth whitening, such as alteration in enamel surface morphology, tooth hypersensitivity and gingival irritation. Indeed, the effects of aggressive not-controlled bleaching treatments may cause changes of the tooth surface integrity, compromising the microstructure of enamel crystals and, hence, enhancing a higher susceptibility to demineralization.

The current strategies to avoid the discomfort and damages associated with bleaching treatments involve the nanotechnology employment in enriching whitening gel by means of nano-forms of HA. Indeed, the nanoHA presents a strong affinity with demineralized surfaces due to its capability to fix into pores created by acid attacks and, therefore, to generate a uniform apatite layer re-establishing the enamel structure.

When an imbalance of the demineralization and remineralization processes on the enamel tissue occurs, tooth decays arise. The standard treatment for a carious lesion consists on the removal of the infected tooth structure, followed by its restoration using a dental material. Resin-based composites have become the gold standard in restorative dentistry for their primary role in guaranteeing excellent aesthetics and function. The principal purpose of nanoparticles in restorative dentistry is their application as fillers in nanocomposites, providing a high ductility without strength loss, scratch resistance, enhanced optical, mechanical and



thermal properties. Moreover, dental nanocomposites depict increased aesthetic properties, such as high gloss retention and excellent polishability and adaptability. Among these ameliorated characteristics, noteworthy are those showed by the new bulk-fill composites (BFC). Indeed, due to their novel monomer content and enhanced curing properties, BFC let to rapidly obtain a satisfactory adaptation of the material into the cavity, decreasing the chair-time needed, also ensuring adequate physical surface features.

Furthermore, thanks to these improved properties of BFC, new filling and modelling techniques have been analyzed and proposed, allowing to realize reliable and simplified restorative protocols.

Therefore, the nanotechnology application supports and facilitates the clinician's needs, which require rapid, safe and repeatable clinical protocols, to obtain the best result in a single appointment. Moreover, in an era marked by Covid-19, the scientific research value applied to daily practice that can optimize working times plays an even more fundamental role.

In summary, the ultimate aim of this PhD thesis is to investigate and evaluate the application of nanotechnologies in clinical practice, with particular attention to Preventive, Aesthetic and Restorative dentistry.



1. Nanotechnological Application in Preventive Dentistry

The recent introduction of nanotechnology has undoubtedly improved the clinical performance of specific procedures to preserve a healthy mouth, thanks to the superior characteristics of the available biomaterials. Furthermore, preventive dentistry has always been considered of primary importance and exceptional interest for the clinician. Considering the advances in nanotechnology and tissue engineering, it has been the protagonist of a major clinical revolution toward the control and management of bacterial biofilms and the efficient repair and regeneration of injured or compromised teeth and their supporting structure. Teeth are organs composed of unique hard tissues, enamel, dentin and cementum, as well as of soft tissues, dental pulp and periodontal ligament (132). Enamel is the hardest and most highly mineralized tissue in the body and is the superficial layer that covers the dental crown, representing the protective shell of the tooth. The enamel structure is composed by more than 96% inorganic material crystals of calcium hydroxyapatite (HA) that renders enamel very strong (133). Dentine represents the hydrated mineralized tissue below the enamel layer, forming the bulk of the tooth. Dentine is 70% inorganic, 20% organic and 10% water by volume (125). Its mineral phase is composed by HA plates, which have lower calcium content and are much smaller than the ones of dentin. The organic component, composed by collagen principally, is a permeable tissue due to the presence of the dentinal tubules, which are a network of small canals that cross all width of dentin, functioning hydraulically during stress transfer (125). Good oral hygiene is essential to maintain the integrity and health of these two structures. Therefore, oral home care represents the first attempt to reduce the incidence of oral diseases, and, among these, the dental caries.



Dental caries is a biofilm-mediated, diet modulated, multifactorial, non-communicable, dynamic disease resulting in net mineral loss of dental hard tissues (51,140). It is determined by biological, behavioral, psychosocial, and environmental factors. As a consequence of this process, a caries lesion develops (113). Dental caries is often cited as the most prevalent chronic disease in children and adults worldwide (50,54,139). Globally, it is estimated that three billion people have dental caries, and an estimated 60–100% of children and adults suffer from caries in their lifetime (122). Furthermore, considering that untreated caries in permanent teeth affected 2.5 billion people, whereas untreated caries in deciduous teeth affected 573 million children (89), dental caries still remains the principal challenge for clinicians. Fortunately, tooth decays are highly preventable and reversible at early state, and the halt of enamel and dentin demineralization is possible with inhibition of biofilm formation (155) and salivary protective factors (160). In this light, nanotechnology provides several new strategies to improve the primary home care tools and clinical preventive treatments, overcoming all concerns related to oral health.

1.1 Remineralizing agents

The modern concept of the caries forming process is based on the recurrence of various cycles of demineralization and remineralization, inducted by acid-producing bacteria in the oral micro-environment (112,166). When there is a loss of minerals from dental hard tissue, before the cavitation, demineralization occurs. The enamel surface demineralization still represents a principal concern and appears to be the main responsible for dentin hypersensitivity (DH) and dental caries insurgence. Conversely, remineralization happens when the calcium and phosphate gradients invert and release towards inside of the lesion (126). Natural remineralization in the initial phase is stimulated by saliva, which contains calcium ions,



phosphate ions, buffer agents, fluoride and other substances (78). However, when this favorable condition is insufficient, topical use of nanomaterial-based agents become crucial to prevent the demineralization. Furthermore, the progression of dental decays is a slow process, and, if applied at early stages, non-invasive procedures play an essential role to block this demineralization, converting the lesion to active state from an inactive state (144,173), hence, increasing remineralization (106).

Nowadays, several remineralizing agents, based on fluoride, calcium, and phosphate ions, in different forms and concentrations, have been introduced for caries prevention and for remineralizing calcified dental tissues. The most commonly and widely organic nanocomplex used are casein phosphopeptide (CPP) stabilized amorphous calcium phosphate (ACP) with a diameter of 2.12 nm (145,164). This milk product facilitates remineralization, inhibits demineralization, and prevents dental caries by forming a calcium phosphate reservoir (116). Indeed, casein phosphate that occurs in CPP-ACP stabilizes calcium and phosphate and facilitates the formation of calcium phosphate nano-complexes on the tooth surface. These compounds act as a source of minerals for the remineralization process. Subsequently, ACP forms and localizes on the tooth surface and acts as a source of calcium and phosphate ions. This material could also quickly turn into HA, which is then deposited on the tooth surface (141,143). Several studies affirm that CPP-ACP is anticariogenic and capable of reversing the early stages of enamel lesions in vitro and in clinical research (112). Also, it is reported that CPP-ACP and flour combination (ACFP) create a synergic effect in caries prevention (76,114). Therefore, the literature confirms that demineralization can be countered or reversed through different CPP-ACP agents in non-cavitated carious lesions (61).

Conversely, the role of fluoride in the prevention and treatment of erosion and teeth demineralization has long been questioned (19,75). The fluoride provides of a globular CaF2-like layer, which, by its dissolution under erosive conditions, briefly protects the underlying



dental tissue (55). Indeed, the presence of fluoride ions in the oral cavity causes the fluorapatite to precipitate the calcium and phosphate ions existing in the saliva. The increase in pH will therefore lead to the formation of larger crystals resistant to fluorine-containing acids (fluoroHA), developing a strong surface layer which increases the resistance to demineralization (18). Indeed, fluoroHA is a more resistant compound than the HA. However, products containing calcium, phosphate and fluoride in their bio-available forms have claimed to increase remineralization over products containing only fluoride (173). Although the topical fluoride agents were shown to be effective in slowing down or halting the progression of dental caries (44), it is well-known that its combination with other nanomaterials provides a reliable and effective treatment to enhance remineralization and prevent tooth decays.

At this regard, the efficacy of different remineralizing agents, based on several forms and concentrations of fluoride, calcium, and phosphate ions, was tested in the Clinic of Dental School, Polytechnic University of Marche [Paper I]. Among the agents tested, qualitative Scanning Electron Microscopy (SEM) evaluations demonstrate that topical applications of CPP+ACP associated with sodium monofluorophosphate could represent the most effective protocol for re-establishing the intact enamel structure, given acid environment.

Furthermore, the polishing rubber cups for dental prophylaxis containing fluoride nanoparticles were analyzed for its clinical use as possible remineralizing agents [Paper II]. Indeed, dental prophylaxis involves the placement of pumice or an abrasive paste in a rubber cup to the clinical crowns of the teeth, in order to remove plaque, salivary pellicle and extrinsic stains before fluoride application [39]. The simultaneous action of cleaning/smoothen dental surface, with the fluoride adjunction, has resulted helpful in the inhibition of dental caries as well as in the prevention of secondary caries in the restored tooth, due to increased fluoride bonding with the enamel surface, thus enhancing its anti-cariogenic property. According to this, the result of this study has showed that the use of topical fluoride within the polishing rubber cup, as routine



part of the final step of many restorative procedures, allows to remineralize enamel and contemporary polish the restoration, simplifying the clinical practice.

In conclusion, the importance of oral health for patients has increased steadily in the number of remineralization agents, products and procedures over recent years. The nanotechnological employment of topical remineralization agents provides a real and valid option to promote enamel remineralization and/or reduce demineralization processes, being considered an interesting and growing area of research in preventive dentistry.

1.2 Anti-inflammatory agents

Preventive dentistry not only deals with the remineralization and protection of hard dental tissues, but it is also devoted to the maintenance of healthy periodontal tissues, controlling plaque accumulation and gingival inflammation and, consequently, preventing the periodontal disease. The supragingival and/or subgingival microbial biofilm can lead to the gingival inflammation, increasing the risk factors that favor periodontal disease progression. The main etiological factor underlying periodontal disease is the presence of anaerobic Gram-negative bacilli residing in the biofilm adhered to the tooth surface (65). Moreover, the biological processes including inflammation and cellular oxidative stress may contribute to the clinical relevance of the onset and state of periodontitis (17). Inflammation and oxidative stress conditions occurring in gingivitis before, and then in periodontitis, and their role in the mechanism of alveolar bone destruction has been claimed by many authors (36,153,161). Therefore, the prevention of gingival inflammation represents the principal concern for the clinician.

In that regard, nanotechnology offers novel strategies for preventive dentistry, especially in the control and management of bacterial biofilms, providing nanoparticles with anti-bacterial



activity. Indeed, the nanosized apatite particles, contained into the anti-inflammatory agents, offer a reliable treatment for gingival diseases, counteracting the irreversible binding of bacteria to the tooth surface. Several works have investigated zinc carbonate HA nanoparticle clusters, which decrease bacterial adherence and reduce initial biofilm formation (22,66). Furthermore, for dental maintenance treatment, both the toothpaste or rinsing solution, containing calcium phosphate nanoparticles (with a diameter of 150–200 nm), combined with chlorhexidine and modified by carboxymethyl cellulose to increase the adhesion properties, offer both mineralizing and antibacterial properties (94).

In addition, it is claimed that the antimicrobial features of disinfectants and antiseptics represent the best choice for chemical plaque control. The well-known chlorhexidine represents the compound with the greatest bacteriostatic and bactericidal features, and it's today widely used in different form and concentration. Nevertheless, there have been many reported side effects, generally when used for long periods, such as taste alteration, supragingival calculus formation, extrinsic tooth staining and desquamation of oral mucosa (102). Moreover, chlorhexidine has been shown to possibly induce cytotoxic and genotoxic effects in cells (109).

Therefore, a widely variety of mouthwashes has been widespread for long period application and has led research into the direction of finding effective and safe products, with a greater focus on herbal drugs. Indeed, the plant extracts can be used as an alternative to chlorhexidine, since their polyphenols compounds exhibit antimicrobial effects (28,134).

In this light, the randomized clinical trial directed in the Outpatient Department of Clinical Sciences and Stomatology of the Polytechnic University of Marche, aimed to assess the anti-inflammatory efficacy and the incidence of adverse effects of an all-natural polyherbal mouthwash in patients with periodontitis, after 3 months of use [Paper III]. The results obtained highlighted that the polyherbal mouthwash application for 3 months showed notable improvement, significantly reducing inflammation and plaque accumulation, providing a



beneficial effect in patients with moderate or severe periodontitis. Furthermore, noteworthy is that no adverse events or side effects were reported or observed in both groups at each time period.

In addition to all-natural polyherbal mouthwashes, desensitizing toothpastes have been tested to reduce gingival inflammation, over to their effective capability to neutralize the dentin hypersensitivity (DH) (129). The DH refers to the rapid and brief onset of a sharp pain provoked by an external stimulus to the dentin exposed. The external stimuli such as those related to temperature, blowing, mechanics, chemicals or osmotic are typical of DH (73), which cannot be associated to a defect or any other particular tooth lesion insurgence. Moreover, previous studies have demonstrated that 46 % of people aged 18–77 years manifests DH (152), in particular younger populations can be especially affected, with up to 42 % of the 18–35-year-old population experiencing sensitivity (180).

The desensitizing toothpaste agents include potassium salt, fluorides, oxalates or bioactive glass in different form and concentration (131,156). Among these toothpastes, desensitizing agents such as calcium sodium phosphosilicate bioactive glass (CSPS) and zinc-carbonate-hydroxyapatite nanostructured microcrystals (CHAs) have been shown to be beneficial even in reducing gingival inflammation.

In this regard, another study conducted by our research group, aimed to evaluate the anti-inflammatory effect and patient appreciation score (VAS) of two toothpastes containing CHA and CSPS desensitizing agents [Paper IV]. CHA and CSPS were compared with an anti-inflammatory and antibacterial herbal based toothpaste (HB). By results obtaining, only CSPS displayed a similar anti-inflammatory effect compared to HB. Despite the low VAS score, CSPS could be considered as a reliable and effective toothpaste in subjects with both dentin hypersensitivity and inflamed gums, highlighting its helpfulness in clinical practice.



Therefore, the successful and promising results showed by toothpastes and/or mouthwashes enriched by nanomaterials clearly highlight its real role in contrasting the gingival inflammation, over its desensitizing capability, despite further investigations, with a large sample size, are needed.

1.3 Prevention of oral diseases

Undoubtedly, a further crucial aim of the prevention in dentistry is represented by the diagnosis of potentially malignant disorders (PMD). The main clinician concern is the timely detection of suspicious clinical lesions to prevent the insurgence of oral disease as the oral cancer. Indeed, the possibility of making early diagnosis is fundamental to decrease high mortality rate and morbidity of oral cancer (178). Oral cancer is the eighteenth most common malignancy in the world, corresponding the 2.0% of the total cancer cases (15). The most common head and neck malignancy is the oral squamous cell carcinoma (OSCC) (178). Despite advances in therapies, the 5-year overall survival rate has remained unchanged in recent decades, mainly due to delayed diagnosis (Gomez et al., 2009). In the last years, several light-based detection systems (LBDS), based on optical properties of biological tissues, have improved the oral mucosal examinations and facilitated the detection of PMD and OSCC. Furthermore, the recent progress of nanotechnology provides new opportunities to detect PMD and OSCC at an early stage (El-Sayed et al., 2005). Further preclinical studies highlighted the potential to enhance diagnostic accuracy of optical diagnostic technologies (e.g., Raman spectroscopy) or imaging techniques (e.g., Magnetic resonance imaging) (Chen et al., 2018). Among the latter techniques, reflectance confocal microscopy seems to improve the evaluation of oral lesions, by detecting backscattered light from illuminated tissue, producing high resolution tissue map.



However, several technological limitations need to be resolved to validate diagnostic accuracy (Lucchese et al., 2016). LBDS showed several advantages compared to the aforementioned approaches, such as low cost and ease of use. For these reasons, it is fundamental to conduct studies to evaluate the efficacy of nanotechnology implementation in LBDS. In this light, thanks to the collaboration with the Oral Pathology Unit of our Dental School, the several LBDS currently employment, providing clinicians with a better understanding of their advantages and limits, and possible future applications of nanotechnologies were reviewed [Paper V]. The promising nanotechnology in the oral diagnostic treatment is based on nanometer-sized semiconductor crystals, called quantum dots (176). Quantum dots coupled with immunohistochemistry (IHC), compared with the traditional one, are more accurate and precise at low protein expression levels and can achieve quantitative detection which could provide much more information for personalized treatment or for cancer identification (47). Furthermore, the biophysical characteristics of these particles provide several advantages over conventional dyes and fluorescent proteins. The possibility to link the quantum dots to molecules with the ability to target cancer cells make them ideal for diagnostic applications in detecting PMD and OSCC (26).

In conclusion, the current LBDS showed great potential for screening and monitoring oral lesions, useful also to identify the lesion margins which must be biopsied, thus assisting the surgical management, and improving their survival rate by means of early diagnosis.



Papers I-V

I. Tosco V, Monterubbianesi R, Sparabombe S, Orilisi G, Putignano A, Orsini G (2020) In Vitro Investigation of the Effect of Different Remineralizing Agents on Human Enamel. Mod App Dent Oral Health 4(3). MADOHC.MS.ID.000189. DOI: 10.32474/MADOHC.2020.04.000189.

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III. Sparabombe S, Monterubbianesi R, Tosco V, Orilisi G, Hosein A, Ferrante L, Putignano A, Orsini G (2019) Efficacy of an All-Natural Polyherbal Mouthwash in Patients with Periodontitis: A Single-Blind Randomized Controlled Trial. Front. Physiol. 10:632. doi: 10.3389/fphys.2019.00632

IV. Monterubbianesi R, Sparabombe S, Tosco V, Profili F, Mascitti M, Hosein A, Putignano A, Orsini G (2020) Can Desensitizing Toothpastes Also Have an Effect on Gingival Inflammation? A Double-Blind, Three-Treatment Crossover Clinical Trial. Int. J. Environ. Res. Public Health Dec 1, 17, 8927.

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LUPINE PUBLISHERS

Modern Approaches in Dentistry and Oral Health Care

DOI: 10.32474/MADOHC.2020.04.000189

ISSN: 2637-4692

Research Article

In Vitro Investigation of the Effect of Different Remineralizing Agents on Human Enamel

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Received: March 03, 2020 **Published:** July 13, 2020

Abstract

This in vitro study aimed to qualitatively evaluate the effect of different remineralizing agents on the enamel pattern, after exposure to an acid solution. Fifteen human sound extracted third molars were collected and randomly allocated into the following five groups: Control Group (CG), without treatment; Negative Group (NG), treated with lactic acid solution; Mousse Group (MG), treated using a mousse of casein phosphopeptide (CPP) and amorphous calcium phosphate (ACP), associated with 0,33% sodium monofluorophosphate; Biosmalto Group (BG), treated with a dentifrice containing amorphous calcium phosphate functionalized with fluorine and carbonate-coated with citrate; Duraphat Group (DG), treated with a 5% sodium fluoride varnish. While CG remained immersed in chloramine solution for the entire period of the study, all the other groups were immersed in lactic acid for 2 hours and then subjected to the appropriate treatment for additional 2 hours, repeating the protocol 3 times a day, for 8 days. Finally, all samples were examined by Scanning Electron Microscope (SEM). SEM micrographs showed that CG had the typical morphological features of sound enamel, whereas NG presented superficial irregularities with early pattern of demineralization, characterized by apparent destruction of enamel prism cores. MG showed intact enamel prismatic arrangement and slight interprismatic dissolution, while BG and DG presented not defined margins of enamel prims and partial loss of interprismatic substance, being more pronounced in DG. Qualitative SEM evaluations demonstrate that topical applications of CPP+ACP associated with sodium monofluorophosphate could represent the most effective protocol for re-establishing the intact enamel structure, given acid environment.

Keywords: Dental caries; Dental enamel; CPP-ACP; Fluoride varnish; Sodium fluoride; Tooth demineralization; Tooth remineralization

Introduction

Dental caries remains a fundamental concern of today's practice and continues to be one of the most common diseases in the world, despite the significant progress of research and the diffused home-practice of oral hygiene [1]. The current concept of the caries forming process is based on the accumulation of numerous episodes of demineralization and remineralization, initiated by acid-producing bacteria in the oral micro-environment [2,3]. The demineralization of dental hard tissue still represents a growing problem and appears to be the main responsible for dentinal hypersensitivity and dental caries insurgence. Demineralization occurs before cavitation when there is a loss of minerals from hard dental tissues. Conversely, remineralization occurs when the calcium and phosphate gradients invert and spread towards inside of the lesion [4]. Natural remineralization in the initial phase is stimulated by saliva, which contains calcium ions, phosphate ions, buffer agents, fluoride and other substances [5]. However, when this ideal condition is lacking, topical agents become essential to avoid the demineralization. Nowadays, the topical fluoride plays a crucial role for caries prevention and for remineralization of the dental structure. The presence of fluoride ions in the oral cavity causes the fluorapatite to precipitate the calcium and phosphate ions existing in the saliva. The increase in pH will therefore lead to the formation of larger crystals resistant to fluorine-containing acids (fluorohydroxyapatite), developing a strong surface layer which increases the resistance to demineralization [6]. However, products containing calcium, phosphate and fluoride in their bio-available

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Mod App Dent Oral Health. *Volume 4 - Issue 3* **Copyrights @** *Giovanna Orsini, et al.*

forms have claimed to increase remineralization over products containing only fluoride [5]. Among these, the remineralizing agent which has been extensively studied and accepted is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP). CPP-ACP is a new agent for the prevention of dental caries [7]. This milk product facilitates remineralization, inhibits demineralization, and prevents dental caries by forming a calcium phosphate reservoir [8]. Indeed, casein phosphate that occurs in CPP-ACP stabilizes calcium and phosphate and facilitates the formation of calcium phosphate nano-complexes on the tooth surface. These compounds act as a source of minerals for the remineralization process. Subsequently, amorphous calcium phosphate forms and localizes on the tooth surface and acts as a source of calcium and phosphate ions. This material could also quickly turn into hydroxyapatite which is then deposited on the tooth surface [9,10]. Several studies affirm that CPP-ACP is anticariogenic and capable of reversing the early stages of enamel lesions in vitro and in clinical research [2]. Also, it is reported that CPP-ACP and flour combination (ACFP) create a synergic effect in caries prevention [11,12]. Therefore, the literature confirms that demineralization can be countered or reversed through different remineralization agents in non-cavitated carious lesions, although there is confusion and inaccuracy of their potential applications. Therefore, it is of great importance to study new strategies and to know different agents to improve the remineralization process. For this reason, the present in vitro study aims to qualitatively evaluate, by means of scanning electron microscopy (SEM), the effect of different remineralizing agents on enamel morphology after exposure to an acid solution.

The following null hypotheses were tested:

a. The remineralizing agents do not influence the demineralized enamel surface after acid environment exposure and

b. There is not difference on the effect of the three remineralizing agents.

Materials and Methods

Fifteen sound third molars were extracted and collected for the present study. The exclusion criteria consisted of presence of lesions and decays, including white spots or hypomineralized lesions, fracture of either crown or root, developmental defects or any other crown deformities. After the surgical extraction, the soft tissue debris were cleaned using an ultrasonic scaler with distilled water. After that, the samples were cut horizontally using a diamond disk coupled to a handpiece, sectioning the crown from the root, and then stored in a 0,5% (w/w) chloramine solution (NH_2Cl). Afterwards, the samples were randomly assigned into the following five groups (n=3): Control Group (CG), without treatment; Negative Group (NG), treated with demineralizing solution (pH=4.4); Mousse Group (MG), treated using GC Tooth Mousse (Recaldent, GC Europe) a mousse of casein phosphopeptide (CPP) and amorphous calcium phosphate (ACP), associated with 0,33% sodium monofluorophosphate; Biosmalto Group (BG), treated with Biosmalto (Curasept S.p.A., Italy), a mousse containing amorphous calcium phosphate functionalized with fluorine and carbonate-coated with citrate; Duraphat Group (DG), treated with Duraphat (Colgate-Palmolive), a 5% sodium fluoride varnish. While CG remained immersed in chloramine solution for the entire period of the study, changing the chloramine solution every 4 days, all the other groups were immersed in a demineralizing solution containing a 0.1M acid lactic, adjusted to pH 4.4, for 2 hours [13], and then subjected to the appropriate treatment for additional 2 hours, repeating the protocol 3 times a day, for 8 days. Finally, after treatment procedures, all samples were carefully cleaned, dehydrated and gold sputter-coated for evaluation using a SEM (Scanning Electron Microscope Zeiss Supra 40, Department of SIMAU, Polytechnic University of Marche, Italy), operated at 30kV and at a working distance of 12mm. Five pictures for each sample at different magnification were performed (4000x and 10000x).

Results

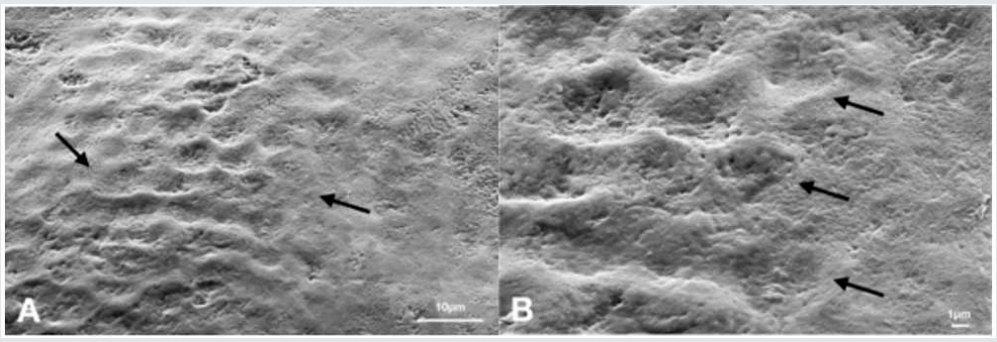

Figure 1: Scanning electron microphotographs of a representative CG Group tooth sample at (A) 4.0K, and (B) 10.00K original magnification. In (A), the typical aspect of sound enamel morphology, with enamel-like crystal structure (black arrows) is showed. In (B), a homogeneous smoothed area between the interprismatic rods (black arrows) is highlighted.

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Scanning electron micrographs, taken at different magnification in the 5 groups, showed dissimilar morphological features of the enamel surface, as follows.

CG displayed the characteristic sound enamel morphology with intact enamel rods and interrods (Figure 1), while NG presented an irregular surface with early pattern of demineralization, characterized by interprismatic tissue dissolution without apparent destruction of enamel prism cores but retained periphery (Figure 2). MG showed a prismatic disposition of the native enamel and a slight interprismatic dissolution. A newly-formed layer of needle-like crystals has been deposited on the surface of the demineralized enamel. These crystals seemed to be randomly arranged, parallel to the surface, reconstructing a structure which is like the intact enamel unit (Figure 3). BG showed an incomplete newly-formed layer of needle-like crystals. The disposition of these latter crystals was rather irregular, thus presenting a partial prisms dissolution which does slightly resemble the native enamel unit (Figure 4). Finally, the DG presented undefined margins of enamel prisms and partial loss of interpretismatic substance, displaying several areas of disorganized arrangement. Therefore, DG results show a partially demineralized surface with no evident signs of reorganization of the native structure of the sound enamel (Figure 5).

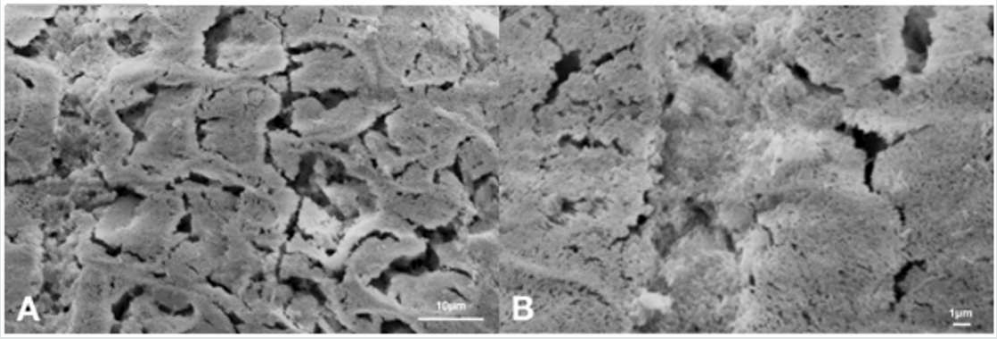

Figure 2: Scanning electron microphotographs of a representative NG Group tooth sample at (A) 4.0K, and (B) 10.00K original magnification. The typical aspect of enamel demineralization. After exposed to the acid lactic solution, the enamel morphology presents an early stage of prisms destruction, with slight interprismatic tissue dissolution occurred at the peripheral areas of the prisms. The margins of the prisms are not clearly identifiable.

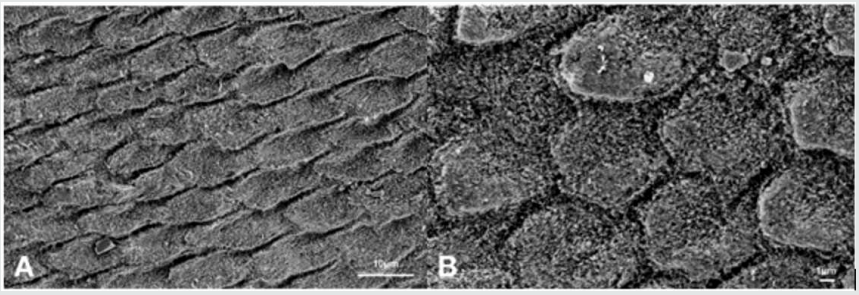

Figure 3: Scanning electron microphotographs of a representative MG Group tooth sample at (A) 4.0K, and (B) 10.00K original magnification. The intact enamel prismatic arrangement and slight interprismatic dissolution. Presence of the ideal oriented and ordered mineralization pattern partially accomplished, with the formation of mineral crystals parallel on enamel surface.

Discussion

The understanding of the imbalance between demineralization and remineralization process is the key factor for caries control and prevention [4,14]. Several studies confirm that an effective management of carious lesions is to early detect the initial demineralization and to use non-invasive techniques by means of various remineralizing agents [15]. A lot of products present in the market, containing calcium, phosphate and fluoride in their bioavailable forms, have claimed to develop and increase the remineralization process. The objective of this in vitro study was to qualitatively evaluate the remineralization effect of three different agents on the enamel surface after exposure of acid environment: GC Tooth Mousse+sodium monofluorophosphate, Biosmalto mousse and Duraphat varnish. the results of this study rejected both of the null hypothesis that the remineralizing agents does not effect the demineralized enamel surface after acid environment exposure and there is not different between on the effect of three remineralizing agents. Since it is difficult to reproduce the demineralization phase in the oral environment in

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vitro, several studies have developed different demineralization methods using pH cycle and acid-etched models, to obtain an artificial enamel lesion [3,16-18]. This study, in agreement with the report of Bonetti et al., shows that an exposure under 0.1M acid lactic (pH 4.4) can reproduce the effects of the demineralization occurring on the enamel surface, thus simulating the initial step of a carious lesion [13]. In fact, the scanning electron micrographs of NG demonstrate an early pattern of demineralization with evidence of interprismatic tissue loss. In particular, the demineralized enamel surface exhibited a loss of the enamel prism core, without preserving the periphery, leading to a disordered arrangement of the rod-like residual [19]. Therefore, to oppose these effects, a lot of products present on the market, containing calcium, phosphate, synthetic hydroxyapatite and fluoride in their bioavailable forms, have been claimed to enhance the remineralization process [20-22]. The null hypotheses of the present qualitative evaluation on the effects of the tested three commonly used remineralizing agents, on enamel surface, after exposure of acid environment, were both rejected. Indeed, MG, BG and DG do influence the superficial enamel demineralization and there is a difference among the effects exerted by the three products. MG (CPP-ACP associated with 0,33% of sodium monofluorophosphate) showed the most promising results. Indeed, SEM images of MG seem to reveal an attempt to recover tissues at the level of the superficial enamel layers, with a predominantly intact and regular interprismatic structure (Figure 3). Moreover, the electron scanning observations displayed the formation of oriented and ordered mineral crystals parallel to the enamel surface. Although ideal oriented and ordered mineralization pattern was accomplished partially, it is noteworthy that the creation of these mineral crystals parallel on enamel surface could be attributed to a secondary crystallization [19]. This suggests an excellent remineralizing potential of the MG, also confirmed by several studies, affirming that, given acid environmental conditions, topical applications of CPP-ACP can be effective in increasing enamel remineralization [13,23]. Indeed, CPP-ACP complex, a milk protein derivative, has been proven to have anticariogenic effects [24-26]. Some studies report that its association with fluoride (CPP-ACPF) improves remineralization [6,27]. Numerous authors have shown that there was a consistent increase in remineralization and a decrease in carious lesion depth when combining the latter two [12,28,29]. Jayarajan et al. [5] presented that the added value of fluoride (NaF 0.2%) to CPP-ACP slightly showed more amount of remineralization than CPP-ACP alone. Therefore, the results analyzed in this study confirm the potential benefits of CPP-ACPF, as frequently highlighted by the scientific literature [17,20,24,28].

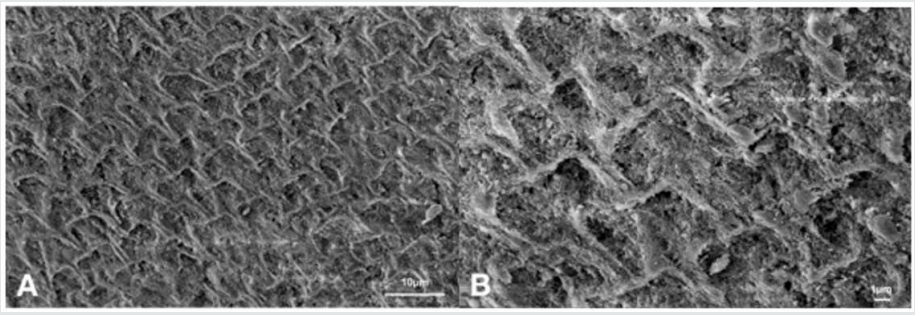

Figure 4: Scanning electron microphotographs of a representative BG Group tooth sample at (A) 4.0K, and (B) 10.00K original magnification. The honeycomb structure was at an early stage, with slight prism cores dissolution. The enamel-like crystal structure reminds a potential remineralizing of the substrate. The new crystal formed on the surface of the demineralized enamel is not evident.

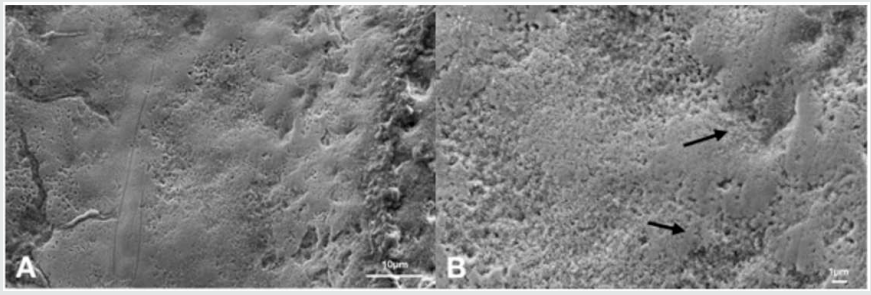

Figure 5: Scanning electron microphotographs of a representative DG Group tooth sample at (A) 4.0K, and (B) 10.00K original magnification. The predominant irregular structure, with slight prism cores dissolution. The margins of the interprismatic structure of the enamel are not evident, presenting a surface disorganization (black arrows).

Citation: Vincenzo Tosco, Riccardo Monterubbianesi, Scilla Sparabombe, Giulia Orilisi, Angelo Putignano, et al. In Vitro Investigation of the Effect of Different Remineralizing Agents on Human Enamel. Mod App Dent Oral Health 4(3)- 2020. MADOHC.MS.ID.000189. DOI: 10.32474/MADOHC.2020.04.000189.



Regarding BG group, it is possible to observe the presence of a partially interprismatic dissolution of the enamel surface. Several studies demonstrated that citrate ions play an important role in stabilizing the precursor ACP phase [30-32]. In addition, [33], showed that the use of fluoride and citrate modify specific ACP and ACPF physico-chemical properties, to generate potentially anti-caries agents and remineralizing effects [33]. Furthermore, by adding citrate to these fluoride-doped ACP nanoparticles, it is possible to restore enamel in its natural structure [31]. ACPF-citrate treated samples showed a faster remineralization effect compared to the ACP- treated ones, especially at the level of enamel prismatic structure, thus being suitable for patients exhibiting severe demineralization [33]. Indeed, in partial disagreement with this latter beneficial effect report, our finding does not clearly demonstrate improved remineralization in BG (Figure 4). Although the newly deposited crystals are less ordered than the natural ones and rather incomplete, it is remarkable that they form a layer of enamel-like crystals, mimicking enamel substrate. In this sense, it would seem possible that the remineralizing action of ACPF combined with citrate is less pronounced than the one exhibited by CCP-ACP associated with 0,33% sodium monofluorophosphate of MG. Among the groups treated with the three remineralizing compounds, DG, in which a 5% sodium fluoride-based varnish was applied, seems to be the one with less evident remineralizing benefits on the superficial enamel, partially confirmed to what is reported by the existing scientific literature [34,35]. Although several studies have assessed the beneficial effects of fluoride varnishes on the remineralization process, Duraphat appears to be the least effective [36-38]. For instance, to prevent the incipient caries, [34] concluded that the varnish containing 5% sodium fluoride associated with CPP–ACP was shown to be more effective than the conventional one based on 5% sodium fluoride [34]. In addition, these data are consistent with [35] that, analyzing three different varnishes, obtained an increase of enamel surface microhardness in samples treated with varnish containing 5% sodium fluoride associated with CPP-ACP [35]. In disagreement with these latter reports and with our findings, some studies claimed that 5% sodium fluoride varnish represents the most effective agent for remineralization [39,40]. Therefore, due to the controversial scientific evidence, the clear and effective advantages of fluoride-based varnishes are still debated, and caution must be paid interpreting the results of the present studies. Scanning electron micrographs obtained by DG displayed a predominantly uneven surface. After the remineralization treatment, several areas of demineralized enamel are still evident, showing a loss of interprismatic substance and disorganized arrangement. Therefore, the attempt to form an ideally-oriented and ordered mineralization pattern on enamel surface has not occurred. The current study design was limited by the in vitro simulation of oral cavity conditions. Another limitation is that Scanning Electron Micrographs do not allow to clearly evaluate the real potential of these products, since only qualitative observations have been obtained. Further studies are needed to quantitively evaluate the changes after treatment, by determining the chemical composition of enamel structure and performing micro-hardness test.

Conclusion

Considering the limitations of this in vitro study, it can be concluded that, in the event of exposure to acid environmental conditions, topical applications of CPP+ACP associated with 0,33% of sodium monofluorophosphate may represent the most effective protocol for re-establishing the intact enamel structure.

Acknowledgement

This work was supported by funds of the Polytechnic University of Marche. The authors would like to thank Adriano Di Cristoforo, Department of Materials, Environmental Science and Urban Planning (SIMAU), CISMIN, Polytechnic University of Marche. He contributed to the setting of Scanning Electron Microscopy for enamel surface analysis. Also, would like to thank Maria Cristina Storaci for samples preparation.

Conflicts of Interest

The authors certify that there is no conflict of interest regarding the publication and no financial relationships relevant to this article.

Author Contributions

V. Tosco contributed to the acquisition and interpretation of data and manuscript discussion. R. Monterubbianesi and S. Sparabombe contributed to the study design and data interpretation. G. Orilisi performed the SEM analysis and contributed to data interpretation. A. Putignano and G. Orsini contributed to the research plan, data interpretation and supervised the entire project. All authors participated to the writing of the present manuscript.

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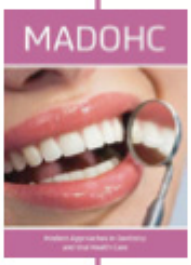

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Dent Res Oral Health 2020; 3(1): 024-034 DOI: 10.26502/droh.0018



Research Article

Effect of a Sodium Fluoride-Releasing Rubber Cup on Hydroxyapatite Crystallinity of Human Enamel: FTIR Spectroscopy Analysis

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Received: 06 February 2020; **Accepted:** 15 February 2020; **Published:** 12 March 2020

Citation: Giulia Orilisi, Riccardo Monterubbianesi, Vincenzo Tosco, Carla Conti, Maurizio Procaccini, Angelo Putignano, Giovanna Orsini. Effect of a Sodium Fluoride-Releasing Rubber Cup on Hydroxyapatite Crystallinity of Human Enamel: FTIR Spectroscopy Analysis. Dental Research and Oral Health 3 (2020): 024-034.

Abstract

Fluoride application is an important strategy to reduce demineralization and enhance remineralization in human teeth. This study aimed to evaluate the effect on enamel hydroxyapatite (HA) crystallinity (C) of a sodium fluoride-releasing rubber cup compared to a non-fluoride rubber cup, using Fourier Transformed Mid Infrared Spectroscopy (FTMIR) analysis. First class direct composite restorations were performed in ten sound extracted molars. Teeth were then divided into two groups (n=5): Control group, polished with



Dent Res Oral Health 2020; 3(1): 024-034 DOI: 10.26502/droh.0018

non-fluoride rubber cup (noFHA), for 10 s; Tested group, polished with a sodium fluoride-releasing rubber cup (FHA), for 10 s. Samples were scraped off on the treated surfaces with abrasive paper and the dust was analyzed using FTMIR. Spectra were acquired in the range of 4000-400 cm^{-1} and each spectrum represented the average of 5 measurements. Phosphate (PO_4) vibration at 604 cm^{-1} was used to analyze C: the full width at half maximum (FWHM) of noFHA was lower than the one of FHA; thus, sodium fluoride, contained in the rubber cup, increased C. The absorption at 1090 cm^{-1} can be considered an additional marker of C, indeed the peak of FHA was higher than the one of HA and noFHA, thus suggesting increasing and stabilizing of the enamel C. In conclusion, the tested sodium fluoride-releasing rubber cup could be an effective polishing system since it not only allows to polish the tooth surface, but also improves the C of enamel HA, thanks to the quick bond done by fluoride.

Keywords: Hydroxyapatite; Enamel Crystallinity; Sodium Fluoride; Dental Enamel; Fourier Transform Infrared Spectroscopy; Polishing System

1. Introduction

Despite all the efforts and advances made in the prevention of tooth demineralization and decays, these pathologies still affect a large number of patients [1]. Dental tissues are continuously undergoing cycles of demineralization and remineralization [2] and caries occurs when demineralization exceeds remineralization [3]. However, the progression of dental decays is a slow process, and, during early stages, non-invasive procedures allow to prevent demineralization, converting the lesion to inactive state from an active state [4, 5], thus enhancing remineralization [6, 7]. In the past decades, various remineralizing agents containing fluoride, calcium, and phosphate ions in various forms and concentrations, were introduced [8-10]. Among these preventive measurements, there are several fluoride-containing products for professional applications with anticariogenic effects [4, 7, 11]. The role of fluoride in the prevention and treatment of erosion and teeth demineralization has long been questioned [12, 13]. Traditional fluoride applications, using monovalent fluorides in low-to-moderate concentrations, such as in toothpastes and mouth rinses, were observed to have preventive effect [12, 14]. Sodium fluoride (NaF) varnish is one of the most concentrated fluoride products commercially available and it has been widely used in Europe for decades [15].

Rubber cups, in combination with prophylaxis pastes, are commonly used for supragingival professional tooth cleaning and polishing. Recently, fluoride-releasing rubber cups were introduced, in order to simultaneously fluoridate the enamel, without a prophylaxis paste, and polish the tooth after a restorative procedure [16, 17]. The two major aspects of fluoride action are: (i) the inhibition of demineralization, and (ii) the enhancement of subsurface remineralization of enamel surface [15]. Fluoride, in the aqueous phase at the apatite crystal surface, plays a determining role in the inhibition of enamel or dentin demineralization [4, 18]. As the pH rises, new and larger crystals that contain more fluoride in form of fluorohydroxyapatite crystals are formed, reducing the enamel demineralization with an



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increase of remineralization [19, 20]. Hydroxyapatite (HA) is composed of calcium, phosphorous, oxygen and hydrogen atoms with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$, characterized by a hexagonal unit cell. Its study and description are of great importance in the field of biomaterials, because HA is the main constituent of bone, enamel and dentine [21]. The crystallinity (C) is correlated to the degree of order within the crystals. In the scientific literature, two ways are reported to measure the C: X-ray diffraction and Infrared Spectroscopy [21, 22]. The ions substitution introduces distortions in the apatite and the C of HA can be modified: in the case of F^-, replacing OH^-, the C is increased, due to the F^- small ionic radius, producing a tiny unit cell volume [23, 24]. The present study is the first report evaluating the effect on enamel hydroxyapatite C of a rubber cup, releasing sodium fluoride at 0.9% used to polish teeth after resin-based composite restorations. The effectiveness of the tested material was compared with a non-fluoride rubber cup, by means of Fourier Transformed Mid Infrared Spectroscopy (FTMIR). The null hypothesis was that sodium fluoride, contained in the tested rubber cup, is able to improve the C of human enamel HA.

2. Materials and Methods

After routinely performed extractions of third molars for orthodontic or prosthetic reasons at the Section of Stomatology of the Polytechnic University of Marche, ten sound teeth were collected. Teeth were washed in an ultrasonic bath with distilled water for 2 minutes in order to remove blood and biological remains, and then carefully examined to exclude the presence of lesions and decays, including hypoplastic defects and cracks; elements exhibiting any of these features were excluded. Afterwards, teeth were stored in artificial saliva. On each tooth, a class I cavity was created using a diamond burr (FG755F-5, Kerr, U.S.) and the restoration performed using a simplified adhesive (Scotchbond Universal Adhesive, 3M ESPE, St. Paul, MN), followed by a nanohybrid resin-composite (Filtek Supreme XTE, 3M ESPE, St. Paul, MN), polymerized with the lamp Elipar DeepCure S (3M ESPE, Seefeld, Germany) for 40 s. Subsequently, teeth were divided into the following two groups (n=5):

- Control group: teeth polished for 10 s, using a non-fluoride rubber cup, with the same shape of the ones, used in the tested group (noFHA).
- Tested group: teeth polished for 10 s, using a fluoride-releasing rubber cup (Pasteless Prophy®, Kerr, U.S.; FHA).

The rubber cup was used with a slow handpiece at 6000 rounds/min, one for each sample. All the procedures were performed by single dental operator to minimize operator changes in variability. Then, the samples were washed with distilled water, dried and analyzed in order to evaluate the C of HA in enamel, using FTMIR spectroscopy [25]. Samples were scraped off on the treated surfaces with abrasive paper, transparent in the infrared, and were analyzed using a Perkin Elmer Spectrum GX1 spectrometer, equipped with a Universal attenuated Total Reflectance (U-ATR) accessory. The spectra were acquired in the range of 4000-500 cm^{-1}, 64 scans and 4

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cm^{-1} spectral resolution, and each spectrum represents the average of 5 measurements.

3. Results

All samples showed comparable spectra in each group in the region 4000-500 cm^{-1} (Figure 1). In order to evaluate C, the vibrations between 1200-500 cm^{-1}, corresponding to the stretching modes of PO_4 of HA, were considered [26]. For data handling, Spectrum v.6.3.1 and Grams AI 7.02 software packages were used. After curve-fitting of the obtained spectra, position, height, full width at half maximum (FWHM) and area under the curves were measured (Figure 2). The most intense band of PO_4 was found at 1090 cm^{-1} in FHA, while, in noFHA it was at 1040 cm^{-1} [27]. Moreover, other spectra were obtained and compared: the spectrum of syntetic HA (pureHA) and the spectrum obtained from the dust, derived from noFHA, mixed with NaF and water, at pH=6, in order to simulate the saliva effects (Mix) (Figure 3).

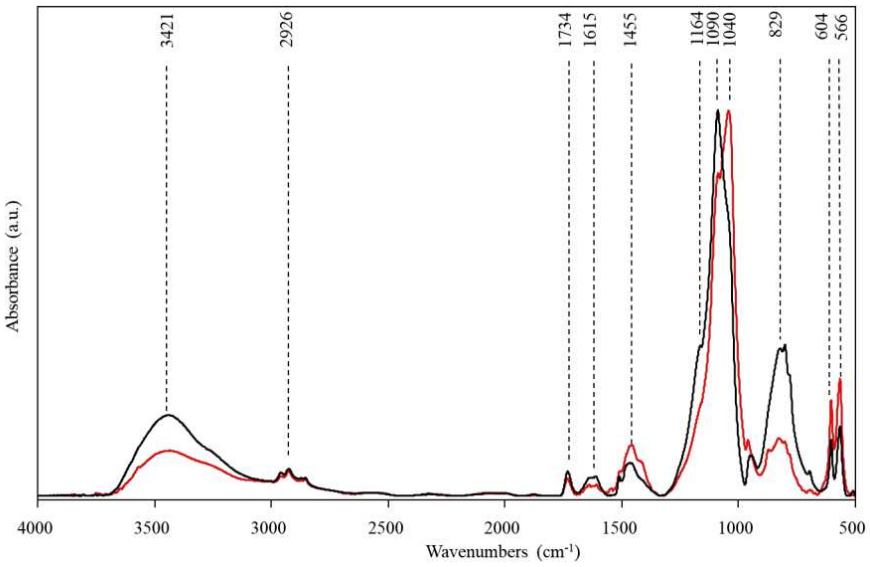

Figure 1: Averaged infrared spectra of noFHA (red) and FHA (black) samples between 4000 - 500 cm^{-1}.

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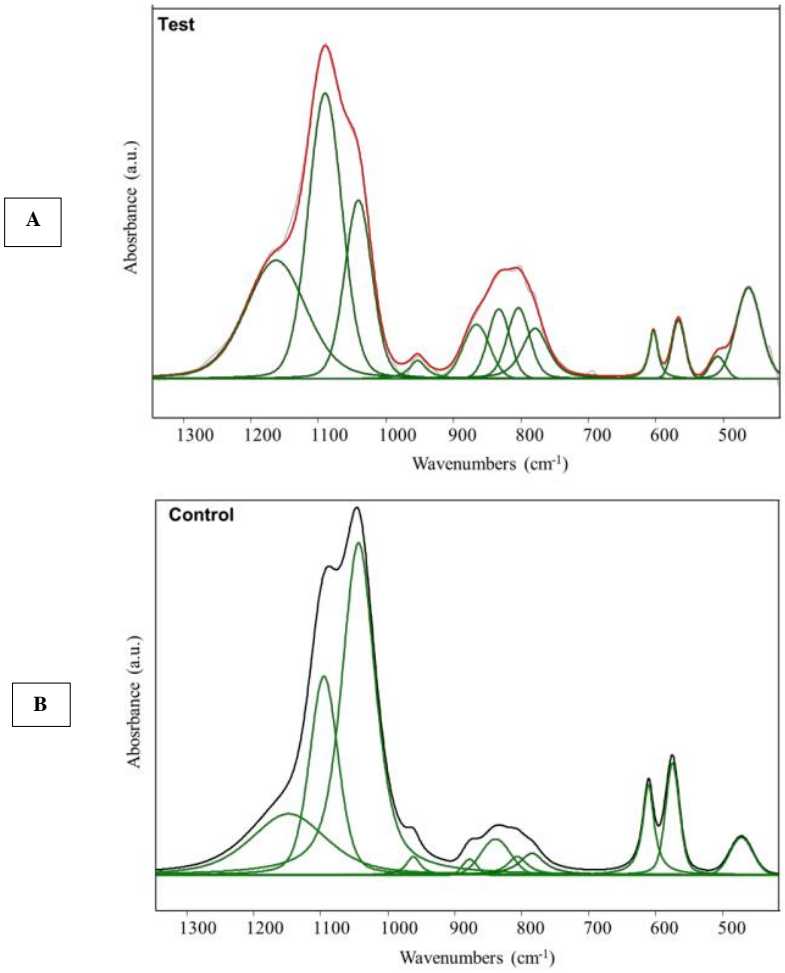

Figure 2: Mixed Gaussian-Lorenzian curve-fitting for FHA (Test, A) and noFHA (Control, B) samples. In FHA (A), the red curve and in noFHA (B) the black one represented the average curve deriving from the obtained spectra.

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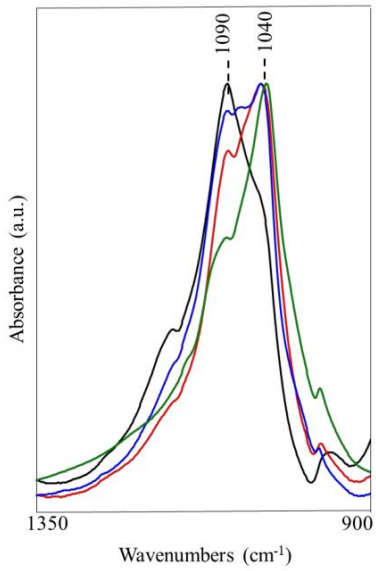

Figure 3: (Blu spectrum): mixture of noFHA dust with NaF and water at pH=6 (Mix); (Red Spectrum): HA (pureHA); (Green Spectrum): noFHA; (Black Spectrum): FHA. The analyzed peaks are found at 1090 cm^{-1} and 1040 cm^{-1}.

4. Discussion

Enamel consists of 96% (wt%) inorganic matrix and 4% (w/w) organic material (mainly lipids and noncollagenous proteins), as well as water, which occupies the free spaces between HA crystals [28]. HA has a hexagonal crystal structure, consisting on isolated PO_4^{3-} tetrahedra connected by a network of distorted octahedra and twisted trigonal prisms, both of which accommodate Ca^{2+}, whereas OH^- are located at the corners [28-30]. FTIR is a technique providing information especially about bonds of molecules. Chemical groups (e.g., hydroxyl, phosphate, amide) can be identified by their specific absorption at different wavenumbers. Because the spectra of these mineral components are quite distinct, vibrational spectroscopy has been extensively used to study all of these tissues, providing information on the nature of the present mineral phases, as well as quantitative information on the changes in mineral and matrix composition as mineralization occurs, and the nature and amounts of substituents in the mineral [25]. In particular, PO_4 vibration at 650-500 cm^{-1} has been used to analyze the C of the apatite domains by FTMIR, using the method proposed by Shemesh et al. [31]. According to his study, the apatite C is inversely proportional to the FWHM of the peak at 604 cm^{-1},



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assigned to the phosphate ions [21, 31-33]. In this study, the FWHM of the peak at 604 cm^{-1} of noFHA is around 22.9, while of FHA is around 16.8. The C of noFHA is lower than the one of FHA; thus, NaF, contained in the rubber cup, results to increase the C of HA, being our results in agreement with the conclusions reported in Shemesh's study [31]. Moreover, the absorption at 1090 cm^{-1} can be considered an additional marker of HA, while the peak at 1040 cm^{-1} is a marker of HA amorphous structure [27, 34]. In Figure 3, the height of the peaks at 1090 cm^{-1} shows different trends. C decreases as follows: FHA>Mix>pureHA>noFHA. This is probably because, using a non-releasing rubber cup during polishing, the amount of HA, contained in enamel, is reduced. On the contrary, if a fluoride-releasing rubber cup is used, fluoride could bind to HA, forming fluorohydroxyapatite, which is a more resistant compound than the HA [35-38]. Thus, as showed in Figure 3, the peak at 1090 cm^{-1} related to FHA is the highest, increasing and stabilizing enamel C. To confirm this finding, the height of the band of FHA at 1040 cm^{-1} is the lowest, because the component of amorphous HA is less than in the other samples. Dental prophylaxis involves the placement of pumice or an abrasive paste in a rubber cup to the clinical crowns of the teeth, in order to remove plaque, salivary pellicle and extrinsic stains before fluoride application [39]. This fact could result in a greater amount of fluoride contacting the enamel surface, thus enhancing its anti-cariogenic property. Therefore, a polishing treatment, such as the one tested in our study, based on the simultaneous action of cleaning/smoothen dental surface, in adjunction with fluoride bonding, can be helpful in the inhibition of dental caries as well as in the prevention of secondary caries in the restored tooth [40]. According to this, the use of topical fluoride within the polishing rubber cup, as routine part of the final step of many restorative procedures, could enormously simplify the clinical practice, allowing to fluoridate enamel and contemporary polish the restoration. Previous studies analyzed the effect on the surface roughness and surface gloss of tooth enamel and composite resin, when exposed to a paste-free prophylaxis polishing cup, as well as a conventional prophylaxis polishing paste [41]. They reported that the conventional prophylaxis pastes increased surface roughness and decreased the gloss of the composite resin and tooth enamel, while the paste-free cups did not significantly affect the surface roughness of the enamel or the restorative materials. According to these last acquisitions it would be interesting to perform a further analysis investigating whether the fluoridated tested polishing cups would affect the roughness and the gloss of the enamel and composite surfaces, as previously performed using other specific polishing systems [42].

5. Conclusions

The null hypothesis that fluoride-releasing rubber cup may improve enamel HA crystallinity was not rejected, suggesting that this polishing system allows to simultaneously polish the restored tooth surface and increase C of enamel HA, without using an additional prophylaxis paste, by quickly bonding the fluoride for stabilizing HA structure. Within the limits of the present study, which is an *in vitro* evaluation with a limited sample size, our findings provide the first demonstration that a fluoride-releasing rubber cup

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may chemically improve HA structure. Therefore, it can be suggested that the tested rubber cups are an effective way for clinicians to polish the teeth after restorative procedures, because they enhance enamel structure strength, being more advantageous, in term of caries prevention, than traditional non-fluoride releasing rubber cups. To better understand the mechanism of action of NaF related to the C of HA, it would be interesting to plan future investigations that will evaluate both *in vitro* and *in vivo* the exact amount of fluoride uptake by enamel.

Acknowledgments

The authors thank Professor Giorgio Tosi for his valuable contribution in drafting this manuscript and Alessandro Lorenzini for samples preparation.

Author Contributions

G. Orilisi and R. Monterubbianesi contributed to the acquisition and interpretation of data and manuscript discussion. V. Tosco and M. Procaccini contributed to the study design and data interpretation. C. Conti performed the FTMIR analysis and contributed to data interpretation. A. Putignano and G. Orsini contributed to the research plan, data interpretation and supervised the entire project. All authors participated to the writing of the present manuscript.

Financial Disclosure

No financial relationships relevant to this article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 22 May 2019
doi: 10.3389/fphys.2019.00632



Efficacy of an All-Natural Polyherbal Mouthwash in Patients With Periodontitis: A Single-Blind Randomized Controlled Trial

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Edited by:
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University of Foggia, Italy

Reviewed by:
Victor E. Arana-Chavez,
University of São Paulo, Brazil
Franz E. Weber,
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Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: 06 March 2019
Accepted: 06 May 2019
Published: 22 May 2019

Citation:
Sparabombe S, Monterubbianesi R, Tosco V, Orilisi G, Hosein A, Ferrante L, Putignano A and Orsini G (2019) Efficacy of an All-Natural Polyherbal Mouthwash in Patients With Periodontitis: A Single-Blind Randomized Controlled Trial. Front. Physiol. 10:632. doi: 10.3389/fphys.2019.00632

Aim: This study aimed to evaluate the anti-inflammatory effect and the incidence of adverse effects of an all-natural polyherbal mouthwash in patients with periodontitis, after 3 months of use. These aims were accomplished by using full mouth bleeding score (FMBS), full mouth plaque score (FMPS), probing depth (PD) clinical attachment level (CAL) and a questionnaire recording any adverse events.

Methods: The present randomized controlled clinical study considered 40 patients with moderate or severe periodontitis, randomized in two groups: a test group (TG) and a control group (CG). TG was instructed to use a polyherbal mouthwash composed of *Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract, and 1.75% of essential oils and the CG was given a placebo mouthwash. Both groups were instructed to rinse for 2 min, twice daily after their routine oral home care with the different mouthwashes. Clinical measurements of FMBS, FMPS, PD and CAL were recorded at baseline (T0) and after 3 months (T1). The incidence of adverse outcomes was recorded at every follow-up. Mann–Whitney U test and Wilcoxon signed-rank test were used for the statistical analysis ($p < 0.05$).

Results: The final study sample consisted of 34 healthy individuals, 17 individuals in each of the two groups. TG and CG showed a statistically significant reduction in FMBS ($p = 0.001$ TG; $p = 0.002$ CG), FMPS ($p = 0.001$ TG; $p = 0.003$ CG), PD ($p = 0.001$ TG; $p = 0.011$ CG) and CAL ($p < 0.001$ TG; $p = 0.020$ CG) values from baseline to 3 months. The TG showed a statistically significant decrease in FMBS and FMPS compared with the CG. No adverse events or side effects were reported or observed in both groups.

Conclusion: The use of polyherbal mouthwash in patients with moderate or severe periodontitis has proved safe and effective in reducing bleeding score and plaque accumulation, after 3 months, compared with placebo, although no difference between the two groups were reported on PD and CAL (both improving at T1).

Keywords: mouthwash, all-natural polyherbal, periodontitis, full mouth bleeding score, full mouth plaque score, probing depth, clinical attachment level, randomized controlled trial



INTRODUCTION

Mechanical plaque control has an essential role in the prevention of periodontal disease, however, it is not sufficiently effective alone (Page and Eke, 2007; Sälzer et al., 2015; Tonetti et al., 2015). A meta-analysis provided strong evidence in favor of the use of antimicrobial agents as adjuncts to mechanical plaque control (Gunsolley, 2006). The author highlighted two types of mouthwashes with consistent antiplaque and anti-gingivitis effects: chlorhexidine gluconate and mouthwash containing essential oils, such as menthol (0.042%), thymol (0.064%), methyl salicylate (0.060%) and eucalyptol (0.092%), which are commercially available. Despite the potent bactericidal action of chlorhexidine, there have been many reported side effects, mainly when used for long periods, such as taste alteration, supragingival calculus formation, extrinsic tooth staining and desquamation of oral mucosa (Lang and Brecx, 1986; Eick et al., 2011). Furthermore, chlorhexidine has been shown to induce cytotoxic and genotoxic effects in cells (Liu et al., 2018).

The increasingly widespread use of the mouthwashes for long periods has led research into the direction of finding effective and safe products, with a greater focus on herbal drugs. Plant extracts can be used as an alternative to chlorhexidine digluconate as their polyphenols compounds exhibit antimicrobial effects. A recent systematic review and meta-analysis, out of a total of 9 articles, investigated the efficacy of daily rinsing with a green tea-based herbal mouthwash in terms of plaque index (PI) and/or gingival index (Mathur et al., 2018). They demonstrated that the herbal mouthwash was not significantly different compared to the standard chlorhexidine-based mouthwashes in reducing plaque and gingival inflammation. Three main groups of plant polyphenols (stilbenes, flavonoids, and proanthocyanidins) were found to exhibit activity against caries, periodontitis and candidiasis in pre-clinical studies, however, there was a lack of strong evidence, regarding randomized clinical trials (Varoni et al., 2012).

Another recently published meta-analyses and meta-regression selected 16 studies comparing a notorious mouthwash containing plant-derived essential oils to placebo solution, cetylpyridinium chloride (CPC) and flossing in the proximal area (Haas et al., 2016). The authors concluded that, in patients with gingivitis, a notorious mouthwash was more efficacious for the reduction of plaque and gingival inflammation than mechanical plaque control either alone or in combination with CPC mouthwash. Due to the proven effectiveness of plant extracts against periodontal disease, there are many commercially available mouthwashes which include one or more active ingredients derived from plants. However, most of them also include additional contents that are artificial and chemically synthesized in the laboratory, being therefore not all-natural (Springhouse, 2003). Indeed, some studies report that they are not entirely innocuous (Hammer and Heel, 2012; Azzimonti et al., 2015).

A recently made commercially available mouthwash includes, in addition to essential oils, a combination of other natural products such as *Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract. It contains no artificial or chemically synthesized ingredients and is therefore entirely natural. There exists no randomized controlled trial that supports the safety and effectiveness of its specific formulation.

The aim of this study was to evaluate the safety and anti-inflammatory effect of the latter all-natural, commercially available polyherbal mouthwash in patients with periodontitis, by comparing it with a placebo mouthwash, after 3 months of use. This was accomplished by measuring the differences, between the two groups, in the following clinical outcomes: full mouth bleeding score (FMBS), full mouth plaque score (FMPS), probing depth (PD) level $\geq$ 5 mm and clinical attachment level (CAL) $\geq$ 4 mm (O'Leary, 1967; Ainamo and Bay, 1975). The incidence of adverse outcomes (the probability of the occurrence of side effects must be the same in the two groups) was also recorded using a questionnaire.

MATERIALS AND METHODS

Forty (40) volunteers, age range 20–65 years, were recruited for the current single-blind randomized placebo-controlled clinical trial. This study was performed in the Outpatient Department of Clinical Sciences and Stomatology of the Polytechnic University of Marche, Ancona, (Italy) between February and June 2017. This study was carried out in accordance with the recommendations of the Ethics Committee of the Azienda Ospedaliero-Universitaria Ospedali Riuniti, Ancona (Protocol N. 2017-0087 UN) and was registered in the Australian New Zealand Clinical Trials Registry (number of trial: ACTRN12618001192279). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study Population

After taking a detailed medical history and an initial clinical and radiologic examination, healthy individuals with a minimum of 20 teeth, smoker and no smoker, were selected. Clinical parameters for inclusion were: diagnosis of severe (at least 2 interproximal sites with CAL $\geq$ 6 mm and 1 interproximal site with PD $\geq$ 5 mm) or moderate (at least 2 interproximal sites with CAL $\geq$ 4 mm or 2 interproximal sites with PD $\geq$ 5 mm) periodontitis according to the Page and Eke classification (Page and Eke, 2007).

Exclusion criteria included: the use of antibiotics and anti-inflammatory drugs in previous 6 months; individuals with orthodontic or prosthetic appliances that could interfere with evaluation; individuals with an allergy to any ingredients used in the study; pregnant or lactating females; motor skills disorder.

All eligible volunteers were given oral and written information about the products and the purpose of the study and were asked to sign an informed consent.

Study Design

This clinical study was designed as a randomized, 3-month placebo-controlled, single-blind clinical trial. A single examiner (SS), with more than 8 years of practice, collected the following clinical parameters: bleeding index, using FMBS and PI, using FMPS, on 6 surfaces; PD obtained by counting the number of



pockets equal or greater than 5 mm; CAL recording the values equal to or greater than 4 mm, on 6 surfaces.

The subjects were randomly divided into two groups, through a computer-generated random table (Orsini et al., 2013). A CONSORT-type diagram explaining the design of this study is presented in **Figure 1**. At baseline (T0), after the clinical parameters assessment, all the participants received a thorough scaling and polishing to remove all plaque, stains and calculus, using ultrasonic scalers and hand instruments. For 3 months, a polyherbal mouthwash was prescribed to test group (TG) and a placebo mouthwash to the control group (CG). The polyherbal mouthwash (Pural Colluttorio, Fitomedical snc, Binasco, Milan, Italy) containing *Propolis* resin extract (1:3), *Plantago lanceolata* leaves extract (1:10), *Salvia officinalis* leaves extract (1:1) and 1.75% of essential oils from *Salvia officinalis*, *Syzygium aromaticum* buds, *Mentha piperita* leaves, *Commiphora myrrha* oleoresin and *Pistacia lentiscus* oleoresin, was made indistinguishable by the label's absence in 100 ml opaque brown bottles marked only with the patient's number. The placebo mouthwash was prepared with the following ingredients: 2 ml of glycerin (sweetening agent), cinnamon and vanilla flavoring agents, and brown food coloring, dissolved in 1 liter of distilled water and placed in 100 ml opaque brown bottles marked only with patient's number.

All participants were instructed to perform their routine oral home care (brushing, flossing or interproximal cleaning) twice daily and, immediately after, to rinse with 1 ml mouthwash diluted in a measuring cup (15 ml) of water for 2 min. All patients were instructed to not rinse/eat anything for 30 min after mouthwash use.

Participants were followed up every 20 days and were asked to bring the empty bottles of the tested mouthwash. Each participant was asked to complete a written questionnaire to assess for any adverse effects related to use of mouthwash at day 20, 40, 60, 80 and 3 months, such as a change in taste sensations, soreness and redness of oral mucosa/tongue/gingiva, feeling of dryness, burning or discoloration. After 3 months (T1), the FMBS, FMPS, PD and CAL parameters were re-collected.

Statistical Analyses

The differences of the single clinical parameters (FMBS, FMPS, PD level $\geq$ 5 mm, CAL $\geq$ 4 mm) were evaluated (Cosyn et al., 2013). For the same clinical score, the difference between TG and CG was carried out using the Mann–Whitney U test both at T0 and T1 ($p < 0.05$). Within each group, the differences in all clinical scores between T0 and T1 were evaluated using the Wilcoxon signed-rank test ($p < 0.05$). The difference in the rate of adverse events between the two groups were evaluated using the Fisher exact test for each time period.

RESULTS

Forty subjects were randomized based on the inclusion and exclusion criteria. There were six dropouts: one due to health reasons, four due to personal reasons and one excluded

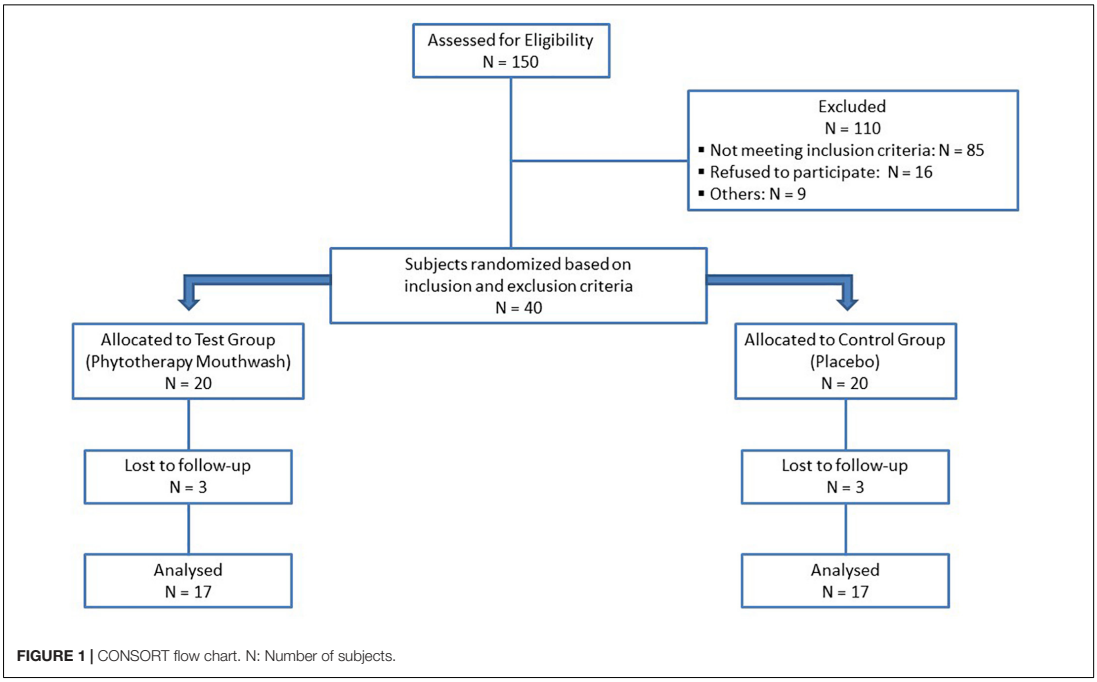

FIGURE 1 | CONSORT flow chart. N: Number of subjects.



because subsequently submitted to a drug therapy. In total, 34 subjects completed the trial (17 each group, see **Figure 1**). **Table 1** presents demographic data of the study population. There were no significant differences in mean age, sex and smoke of individuals between the two groups. At T0, there were no statistical differences in FMBS between the CG and TG; both groups showed a significant reduction in FMBS from T0 to T1. At T1, the TG group showed a statistically significant decrease in FMBS ($p = 0.002$) compared with the CG group (**Table 2**).

At T0, the two groups showed no significant differences in mean of FMPS values, full-mouth PD and CAL. At T1, the TG group showed a significant decrease in FMPS ($p = 0.005$) compared with the CG group. Moreover, there were no significant differences in mean of full-mouth PD and CAL between the two groups at T1: both groups showed a significant reduction in PD ($p = 0.001$ TG; $p = 0.011$ CG) and CAL ($p < 0.001$ TG; $p = 0.020$ CG) values from baseline to 3 months (**Table 2**). There were no subjective complaints, adverse outcomes or side effects reported or observed in both groups at day 20, 40, 60, 80 and 3 months (**Table 3**).

TABLE 3 | Side effects evaluation at each time period (day 20,40,60,80, 3 months) in the test (using polyherbal all-natural mouthwash) and Placebo groups.

SIDE effects		Test group ($n = 17$)	Control group ($n = 17$)
Soft tissue	Redness oral mucosa/tongue/gingiva	0	0
	Soreness oral mucosa/tongue/gingiva	0	0
	Mouth burning	0	0
	Desquamation	0	0
	Oral dryness	0	0
Hard tissue	Brown spots	0	0
Other	Taste perturbation (loss of taste)	0	0

DISCUSSION

The effectiveness of natural products and plant extracts is evidenced by several studies on its use in the treatment of oral diseases (Palombo, 2011; Škrovánková et al., 2012; Chinsembu, 2016). We conducted the first-ever randomized, controlled trial to investigate the effectiveness of a new combination of completely natural plant extracts (*Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract, and 1.75% of essential oils) in a single mouthwash available on the market, by comparing it with a placebo mouthwash.

TABLE 1 | Basic demographic characteristics of study population.

Parameter	Test ($n = 17$)	Control ($n = 17$)
Mean age ± SD (years)	51,05 ± 7,7	51,29 ± 10,2
Age range (years)	30 to 62	30 to 62
Males/females	8/9	8/9
Smokers/no smokers	6/11	6/11

The effectiveness of other herbal mouthwashes compared to a placebo mouthwash in patients with gingival or periodontal disease has been shown. Despite this, the topic is still controversial, due to some studies not using standardized methodology or having very short follow-ups. The effectiveness of three herbal extracts (*Juniperus communis*, *Urtica dioica*, *Achillea millefolium* on plaque and gingivitis) was studied by comparing it to a placebo mouthwash for 3 months, finding no beneficial effects (Weijden et al., 1998). Another study which investigated bacterial enumeration, plaque accumulation and gingival bleeding of a commercial herbal mouthwash containing *Salvadora persica* extract versus placebo over 3 weeks, found a statistically significant decrease in the PI of test subjects; a significant reduction in gingival bleeding was observed in both test and placebo groups (Khalessi et al., 2004).

Formulated in 2001, based on the traditional and historical uses of its functional ingredients and their components,

TABLE 2 | Values (mean ± SD) for Plaque and bleeding index and PD and CAL at Baseline and at 3 months.

Parameter	Interval	Test	Control	*P value*
FMBS	T0	12.23 ± 9.49	9.19 ± 6.23	*0.438*
	T1	1.63 ± 1.74	3.85 ± 2.68	*0.002*
	P value	*<0.001*	*0.002*	
FMPS	T0	44.70 ± 6.36	44.21 ± 16.04	*0.138*
	T1	17.64 ± 5.59	29.70 ± 14.38	*0.005*
	P value	*<0.001*	*0.003*	
PD ≥ 5 mm	T0	8.23 ± 14,42	2.36 ± 3.21	*0.068*
	T1	1.12 ± 1.79	1.37 ± 2.90	*0.820*
	P value	*0.001*	*0.011*	
CAL ≥ 4 mm	T0	43.25 ± 19.65	33.40 ± 16.28	*0.174*
	T1	29.17 ± 17.66	27.30 ± 17.39	*0.708*
	P value	*<0.001*	*0.020*	

Statistically significant at P < 0.05. FMBS, full mouth bleeding score; FMPS, full mouth plaque score; PD, probing depth; CAL, clinical attachment level.



the tested polyherbal mouthwash is all-natural. Numerous studies have highlighted essential oils antimicrobial effects even against multi-resistant bacteria (Mayaud et al., 2008; Chandra Shekar et al., 2015; Chinsembu, 2016). Because of their complex chemical composition, which includes more than 100 different therapeutic compounds, essential oils have a broad biological and antimicrobial activity spectrum: antibacterial, antifungal, and antiviral (Burt, 2004). *Plantago* species have considerable antiviral, anti-inflammatory, and antioxidant activities (Gálvez et al., 2005). *Plantago lanceolata* phenolic compounds seem to play a potential role in the control of bacterial growth and pathogenic oral flora virulence (Smullen et al., 2007). The antimicrobial effect of *sage* extract has been shown experimentally and clinically (Bozin et al., 2007; Geuenich et al., 2008). *Pistacia lentiscus* is an evergreen plant of the *Anacardiaceae* family, commonly found in the Mediterranean region; its fruits, galls, resin, and leaves have a long tradition in folk medicine. Recently, *Pistacia lentiscus* was found to exhibit an anti-inflammatory activity by reduction of inflammatory mediator production and inhibition of leukocyte recruitment to the inflammatory site (Ben Khedir et al., 2016). It also exhibited an antioxidant effect as a source of antioxidant molecules and indirectly as a stimulator of the activity and the expression of an antioxidant enzyme (Ben Khedir et al., 2016). *Propolis* is a well-known resinous material collected by bees; more than 300 components have been identified in *Propolis*, revealing that its composition is dependent upon the plant source and local flora. Several researchers have reported its antibacterial, antiviral, antitumor, anti-inflammatory property and immunomodulatory action (Sforcin, 2016; Oryan et al., 2018).

There is therefore strong beneficial evidence for these individual plant extracts in the tested mouthwash; however, no evidence exists for the combination of these plant extracts, formulated as a polyherbal mouthwash. This may be due to the quality of the substances and its delicate production process. Essential oils are unstable and fragile volatile compounds; they could be easily degraded by either oxidation, volatilization, heat or light if they are not protected from external factors, especially during the collection, storage and processing phase (Asbahani et al., 2015). *In vivo* studies and clinical trials on other herbal mouthwash have been performed (Khalessi et al., 2004; Haffajee et al., 2006), however, the need for clinical trials about the safety and efficacy of the combination of these herbal extracts in the tested mouth has been highlighted (Pan et al., 2013; Freires and Rosalen, 2016).

In the present study, the results are very encouraging; in both groups there was a significant reduction of inflammation from T0 to T1, confirming what is well described in the literature: mechanical therapy (scaling and polishing) improves clinical conditions by lowering the microbial load either by physical removal of plaque and by radical alteration of the subgingival habitat (Khalessi et al., 2004; Haffajee et al., 2006). However, in this study, 90 days after mechanical therapy, the TG had a greater reduction also in inflammation (FMBS) compared to the CG ($p = 0.002$).

A recent study investigated whether the adjunctive use of a mouthwash containing three natural essential oils (*Cymbopogon flexuosus* oil, *Thymus Zygis* oil, *Rosmarinus officinalis*) following sub-gingival debridement and scaling and root planning (SRP) could improve the clinical results regarding changes of the clinical parameters of PD, CAL and bleeding on probing (BoP) (Azad et al., 2016). Significant improvements of AL, PD, and BoP occurred in both groups after 3 months ($p < 0.001$); PD and BoP being significantly lower in the TG. In accordance with Azad study, our trial shows a significant improvement of PD ($P = 0.001$) and CAL ($P < 0.001$) in both groups, which can be explained because each subject in both groups had a preliminary scale and polishing at T0. Contrarily, no difference in PD and CAL between the two groups was revealed. This finding may be explained by the fact that PD reduction and gain in CAL are the expected result of mechanical instrumentation alone (Claffey et al., 1988; Haffajee et al., 1997). Therefore, mouthwashes do not seem crucial for these latter clinical outcomes. In support of our findings, another 3-month double-blind, randomized placebo-controlled trial studying clinical and microbial effects of an essential oil mouthwash in periodontal patients did not demonstrate any significant differences in full-mouth PD and full-mouth CAL between the test and placebo group (Cosyn et al., 2013). Indeed, plaque and bleeding indexes showed a significant reduction over time ($P = 0.029$); but, contrary to our data, there were no statically significant differences between the two groups (Cosyn et al., 2013).

The present single blind randomized controlled study shows a significant PI reduction in the TG and CG group. The considerable improvement of plaque control in both groups could be attributed to the Hawthorne effect; the subjects modified their oral hygiene routine due to the awareness of being observed (McCarney et al., 2007). However, FMPS has a significant difference between the two groups ($P = 0.005$), being significantly decreased in the TG. Our results are in agreement with other studies which investigated the anti-plaque and antigingivitis effects of a commercially available mouthwash containing essential oils with proven efficacy in individuals with and without periodontal disease (Sharma et al., 2004; Cortelli et al., 2009). However, although some herbal mouthwash showed side effects, mainly a mild mouth burning sensation (Manipal et al., 2016), the tested combination of all-natural polyherbal mouthwash has not demonstrated any discomfort or adverse events (**Table 3**). This study highlights the superior efficacy of the tested polyherbal mouthwash, over an essential oil mouthwash, as an agent without side effects, having considerable properties in controlling oral inflammation and microbiota. At this regard, a very recent comparative *in vitro* study demonstrated that chlorhexidine-based mouthwashes are still the most effective in regulating microbial homeostasis (Ardizzoni et al., 2018). However, chlorhexidine has several side effects that must be pondered when prescribing mouthwashes containing this molecule. Indeed, to the best of our knowledge, the need to develop alternative, innocuous but effective solutions are highly motivated. Therefore, despite the limitation that the polyherbal mouthwash was not compared to the "gold standard"



chlorhexidine mouthwash and was only compared to a placebo, its all-natural ingredients have to be kept in great attention, since the preliminary evidence of effectiveness and safety is very promising.

CONCLUSION

Common oral diseases such as gingivitis and periodontitis are based on microorganisms; herbal mouthwashes have an integral role in controlling the oral microbiota, and therefore are a useful adjunct in preventing periodontal disease. The use of the polyherbal mouthwash for 3 months significantly reduces inflammation (FMBS) and plaque accumulation (FMPS), showing a beneficial effect in patients with moderate or severe periodontitis. The most important finding is that, in bleeding and plaque score, participants using the all-natural polyherbal mouthwash showed significant improvement compared with the control. Noteworthy is that no adverse events or side effects were reported or observed in both groups at each time period. Further studies, in the near future, will be planned to compare it with the chlorhexidine mouthwash or other natural mouthwashes with proven efficacy.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the dataset is not publicly available as it is the property of the Polytechnic University of Marche prior to its publication. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Azienda Ospedaliero-Universitaria Ospedali Riuniti, Ancona (Protocol N. 2017-0087 UN) and was registered in the Australian New Zealand Clinical Trials Registry (number of trial: ACTRN12618001192279). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SS and RM contributed to collection of data, conception and design, data analysis and interpretation, manuscript writing, critical reading and editing of the manuscript, and final revision and approval of the manuscript. VT and GuO assembled the data, analyzed and interpreted the data, wrote the manuscript, and approved the final version of the manuscript. GoO and AH interpreted the data, wrote and edited the manuscript, and approved the final version of the manuscript. LF, AP, and GoO contributed to conception and design, statistical analysis and interpretation, manuscript writing, and final approval and revision of the manuscript.

FUNDING

This work was supported by funds of the Polytechnic University of Marche (GoO and AP). The materials for the study were kindly donated by Fitomedical snc, Binasco, Milan, Italy.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Can Desensitizing Toothpastes Also Have an Effect on Gingival Inflammation? A Double-Blind, Three-Treatment Crossover Clinical Trial

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Received: 29 October 2020; Accepted: 27 November 2020; Published: 1 December 2020



Abstract: Background: Many desensitizing toothpastes exist commercially; however, few clinical trials have investigated their anti-inflammatory effects. This study aimed to evaluate the anti-inflammatory effect and patient appreciation of two toothpastes containing desensitizing agents: (1) a zinc-carbonate-hydroxyapatite nanoparticle (CHA) and (2) a calcium sodium phosphosilicate bioactive glass (CSPS). Methods: CHA and CSPS were compared with an anti-inflammatory and antibacterial herbal based toothpaste (HB). The aims were accomplished by comparing the following outcomes: (1) the reduction in plaque and bleeding score (Full Mouth Plaque Score (FMPS) and Full Mouth Bleeding Score (FMBS), respectively); (2) the antibacterial activity (AbA) of the toothpaste by saliva samples; (3) the patient appreciation score (Visual Analogue Scale; VAS). Clinical parameters were assessed at baseline and 14 days post-treatment. Results: The final sample consisted of 25 subjects, aged between 20 and 58 years. Although no differences in FMPS were reported ($p > 0.05$), both desensitizing toothpastes showed an improvement in FMBS. CSPS and HB recorded more AbA compared to CHA ($p < 0.05$). Moreover, HB resulted in a higher VAS score than both desensitizing toothpastes ($p < 0.05$). Conclusion: In conclusion, only CSPS displayed a similar anti-inflammatory effect compared to HB. Despite the low VAS score, CSPS could be considered as a valid and effective toothpaste in subjects with both dentin hypersensitivity and inflamed gums, highlighting its utility in clinical practice.

Keywords: desensitizing toothpastes; CSPS; CHA; anti-inflammatory; anti-inflammatory activity

1. Introduction

Toothbrushing is a complex process, and its efficacy is strongly influenced by the subject's compliance and manual dexterity. In the developed world, most people would not brush their teeth without using a toothpaste [1], with the primary reason being to have a "good taste" in one's mouth after daily oral care [2]. However, toothpaste is an ideal vehicle for different agents used to combat several dental diseases: agents to control gingivitis and plaque accumulation, antibacterial agents used to prevent caries and periodontal disease, tooth whitening agents and desensitizing agents used to alleviate dentin hypersensitivity (DH).

DH is a widespread oral health problem among the adult population [3]. It is characterized by an acute passing pain which affects the exposed dentin in response to different stimuli. Dentin,



and consequently dentinal tubules, can become exposed after gingival recession or other oral insults, such as attrition or erosion, which ultimately lead to a loss of enamel or cementum [4]. Many theories have been proposed to explain the biological mechanism of DH; however, the hydrodynamic theory is the most widely accepted [3]. According to this theory, pain occurs when a stimulus causes the rapid displacement of fluid within exposed open tubules, which in turn excites nerve terminals at the inner ends of the tubules or the periphery of the pulp [3,5]. Restorative treatment can be performed to alleviate or prevent dentine hypersensitivity. In addition, desensitizing toothpastes can be beneficial in managing dentine hypersensitivity.

The active desensitizing agents found in toothpaste include potassium salt [5], fluorides [6,7], oxalates [7] or bioactive glass [8–11]. In recent years, desensitizing agents such as calcium sodium phosphosilicate bioactive glass (CSPS) and zinc-substituted carbonate-hydroxyapatite nanostructured microcrystals (CHAs) have been shown to be beneficial even in reducing gingival inflammation [12–15]. Indeed, toothpastes containing these desensitizing agents, CSPS and CHA, are retailed not only to reduce DH but also gingival inflammation. However, no randomized clinical trial (RCT) has been performed to compare them with an anti-inflammatory toothpaste (herbal based toothpaste (HB)) with proven efficacy. Indeed, the main active natural ingredients of HB reduces plaque accumulation, and consequently, gingival inflammation [16]. For this reason, our double-blind, three-treatment crossover study aimed to compare the anti-inflammatory effect of CSPS and CHA with HB in subjects affected by gingival inflammation.

The aim of the present RCT was accomplished by measuring the differences among three treatment groups at baseline and 14 days post-treatment, in the following outcomes: (1) Full Mouth Plaque Score (FMPS) and Full Mouth Bleeding Score (FMBS) of the participants; (2) the antibacterial activity (AbA) of the toothpastes by evaluating the bacterial vitality in salivary samples; (3) patient's appreciation using Visual Analogue Scale (VAS). Therefore, the aims of this study were: (1) to evaluate the antiplaque and anti-inflammatory properties and AbA of the tested toothpastes compared to an anti-inflammatory and antibacterial HB; (2) to assess the patient's appreciation of the tested toothpastes.

2. Materials and Methods

This study was performed in the Outpatient Department of Clinical Sciences and Stomatology of the Polytechnic University of Marche, Ancona (Italy) between October 2018 and May 2019. Inclusion criteria were: (1) minimum of 20 teeth; (2) good physical health; (3) aged 18 to 75 years; (4) affected by gingival inflammation for the following conditions: plaque-induced gingivitis in an intact periodontium [17]; plaque-induced gingivitis in a reduced periodontium without a history of periodontitis [17]; plaque-induced gingivitis in a periodontally stable patient [17]; gingival inflammation of periodontitis stage I patients and slow rate of progression [18]; (5) provision of written informed consent. The exclusion criteria were: (1) active caries; (2) periodontitis stage III or IV [18]; (3) wearers of dentures and orthodontic appliances; (4) severe malocclusion; (5) use of oral antiseptics in the previous three months; (6) subjects with medical disorders; (7) undergoing antibiotic or other antimicrobial therapy in previous 6 months; (8) the presence of any systemic disease that could alter the production or composition of the saliva or dental plaque; (9) individuals with an allergy to any ingredients used in the study; (10) smoking; (11) pregnancy.

Thirty-one eligible volunteers were recruited for the double-blind three-treatment crossover trial. Six volunteers refused to participate for personal reasons. Twenty-five subjects were used in this study (9 females and 16 males, aged 20 to 58). The protocol consisted of a pre-experimental phase and 3 experimental phases of 14 days, each followed by a 5-day wash-out interval (Figure 1) [19–22].

Int. J. Environ. Res. Public Health **2020**, *17*, 8927

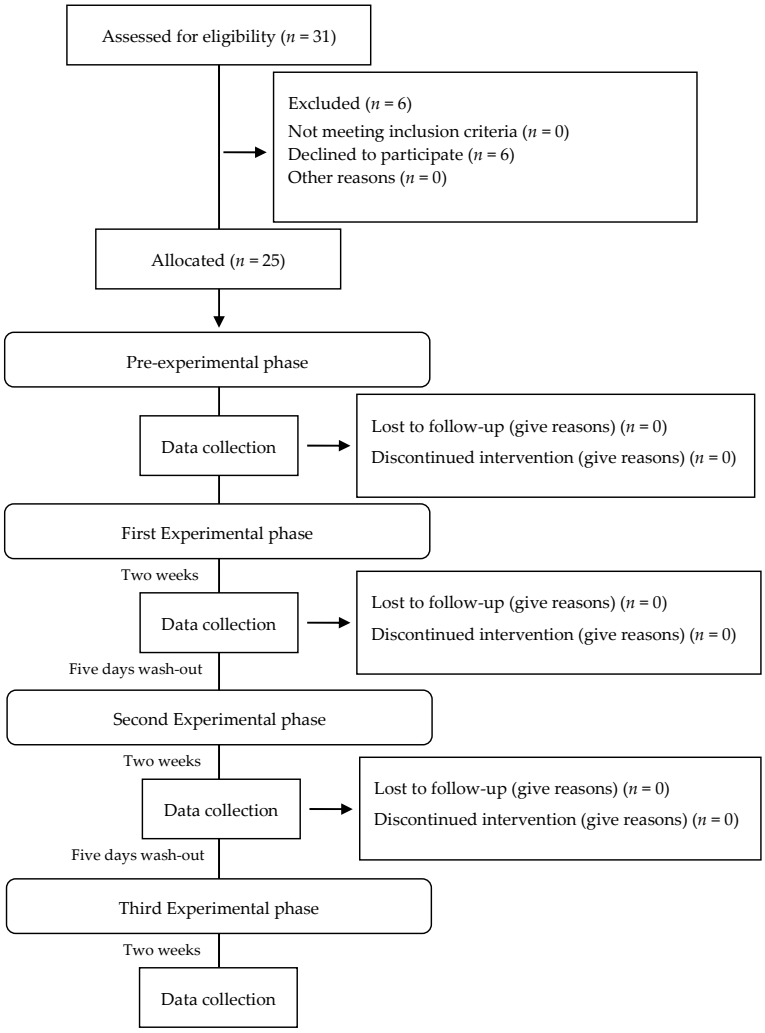

Figure 1. Flow diagram for patient enrollment and study design.

In the pre-experimental phase, performed 14 days prior to the experimental phase, subjects underwent a professional oral hygiene procedure, using a blended approach of both mechanical and manual instruments, followed by polishing and flossing in order to eliminate any existing dental plaque and avoid a possible residual effect of other oral hygiene products they may have used. Written instructions and a new toothbrush (Curaprox CS 820 medium, Curaden GmbH, Kriens, Switzerland) were provided to each subject. In the written instructions, the subject was informed to perform toothbrushing 2 times a day [9] with water only, suspending the use of any oral hygiene aids such as mouthwash, dental floss and proxabrush.

After the pre-experimental phase (T0), baseline values of FMPS and FMBS were recorded, and a saliva sample was taken from each subject immediately after all his/her teeth were polished and flossed. The subjects then received the first tested toothpaste and a new toothbrush. Moreover, they were instructed to brush two times a day with a pea-sized dose of the assigned toothpaste. In addition to



verbal instructions, the subjects received written instructions. Each experimental phase lasted 14 days and was followed by a 5-day wash-out interval in which the subjects followed the same instruction of the pre-experimental phase. After each experimental phase and before the wash-out interval, FMPS and FMBS were measured and salivary samples were taken, and the patient's appreciation score was recorded using the VAS. Each experimental phase was assigned a name according to the toothpaste provided. The order of the experimental phases which was randomly chosen was as follows: HB, CSPS, CHA. The tested toothpastes were made indistinguishable by a white label coating that wrapped around the 25 mL tube. Each subject was asked to return all the tubes (used or not used), at the end of each experimental phase. Before each experimental phase, new toothpaste tubes and toothbrushes were provided to the patients, as described in Nogueira-Filho's study [19]. Additionally, each subject's teeth were polished and flossed. Oral hygiene instructions and dietary advice was given prohibiting the use of chewing gum and candies in both the pre-experimental and experimental phase.

Two expert dental hygienists (SS and FP) performed the data recording at each oral hygiene session of both pre-experimental and experimental phases. FP was responsible for delivering the anonymous toothpastes while SS was responsible for giving the oral hygiene instructions and recording the data at the end of the pre-experimental and each experimental phase. The order in which toothpastes were provided in this double-blind three-treatment crossover study was determined through a computer-generated random sequence. The investigators were neither involved in the randomization process, nor were they aware of the assigned group in all outcome evaluations. The study was conducted in accordance with the Declaration of Helsinki. Moreover, the protocol was carried out in accordance with the recommendations by the local Ethics Committee (N. 0405OR; 25 February 2016).

2.1. Toothpastes

The following two toothpastes containing desensitizing agents were compared with a well-known anti-inflammatory toothpaste:

- CSPS contains 5% calcium sodium phosphosilicate bioactive glass (Sensodyne Protezione Completa, GlaxoSmithKline Consumer Healthcare, Brentford, United Kingdom).
- CHA is a zinc-carbonate-hydroxyapatite nanoparticles toothpaste (Biorepair plus Parodontgel, Coswell S.p.A, Bologna, Italy).
- HB is an herbal anti-inflammatory toothpaste containing sodium bicarbonate (67%), Peppermint, Echinacea, Sage, Myrrh, Rhatany, Chamomile (3%) and sodium fluoride (1400 ppm) (Parodontax, GlaxoSmithKline Consumer Healthcare, Brentford, United Kingdom). It has shown anti-inflammatory activity and therefore was considered as the control [23–26].

2.2. Clinical Assessment

The plaque score and bleeding score of the subjects were measured using the periodontal indices, FMPS and FMBS, respectively. Their clinical assessments were performed on 6 surfaces, as described in the literature [27,28].

The AbA of the tested toothpastes was analyzed by measuring the vitality of salivary bacteria. This was accomplished through 10^{-3} saliva dilution, homogenization using a vortex mixer for 30 s, followed by incubation at 37° for 24 h in Columbia agar +5% sheep blood [29]. The saliva sample was always taken in the morning, before any other assessment, without the subject brushing their teeth or eating. Samples of 2 mL of unstimulated saliva were collected using the spitting method [29]. After each experimental phase, the patient appreciation score (VAS) was assessed by choosing a number on a 10 cm line, with a score of 0 indicating a bad taste and a score of 10 indicating a good taste [30].

2.3. Sample Size Examination

Calculation of the number of subjects was centered on the principal outcome i.e., the average decline in the index of plaque between the beginning and the end of treatment. It is estimated that an



initial average value of plaque index was equal to 55%. Assuming a relative decrease of 15% of plaque index (i.e., 8% absolute), a standard deviation not dissimilar from baseline (10%), and an intra-cluster average correlation (0.2 considering the wash-out) with an alpha equal to 0.05, the minimum sample size to obtain a power of 80% was 22 subjects [22]. In order to prevent disclaimers, the minimum sample size was estimated at 31 subjects.

2.4. Statistical Methods

Statistical analysis was performed with R Project for Statistical Computing 3.3.0 (https://www.r-project.org/) (R Development Core Team, Ames, IA, USA) and Microsoft Excel 2013 (Microsoft, Washington, DC, USA). Statistical evaluations were made only on the subjects who concluded the study. For each clinical outcome, the comparison between the baseline and post-intervention within the three treatment groups was analyzed using the Wilkoxon test, also called "matched-pairs signed ranks test". The comparison between differences in the pre-post intervention mean, for all continuous outcomes, was carried out by a non-parametric Kruskal–Wallis test. A p-value < 0.05 was considered significant for all results.

3. Results

Of the 31 recruited subjects, a total of 25 subjects, 9 female and 16 male, age range 20–58 years, completed the trial. Table 1 shows the results of the study.

Table 1. Comparison between groups. All values are expressed as means and standard deviations.

	T0	HB	CSPS	CHA
FMPS	0.54 ± 0.14 ^{a}	0.55 ± 0.10 ^{a}	0.52 ± 0.10 ^{a}	0.55 ± 0.09 ^{a}
FMBS	0.17 ± 0.06 ^{a}	0.10 ± 0.04 ^{b}	0.09 ± 0.04 ^{b}	0.08 ± 0.03 ^{b}
AbA ($\times 10^{8}$)	20.49 ± 17.53 ^{a}	4.45 ± 3.06 ^{b}	6.25 ± 13.48 ^{b}	14.41 ± 19.66 ^{a,b}
VAS		6.30 ± 2.08 ^{a}	3.00 ± 2.08 ^{b}	3.05 ± 1.88 ^{b}

Note: Full mouth plaque score (FMPS), full mouth bleeding score (FMBS), antibacterial activity (AbA) and patient's appreciation (Visual Analogue Scale; VAS) at baseline (T0) and at the end of each experimental phase. Each experimental phase was assigned a name according to the toothpaste provided, and the order of the experimental phases was as follows: herbal based toothpaste (HB), calcium sodium phosphosilicate bioactive glass (CSPS), zinc-carbonate-hydroxyapatite nanoparticles (CHA). Different superscript letters (^{a}/^{b}/^{a,b}) indicate significant differences between groups ($p < 0.05$).

All the tested toothpastes displayed a similar FMPS ($p > 0.05$). On the other hand, they showed an improvement of FMBS (HB = 0.10 ± 0.04; SPS = 0.09 ± 0.04; CHA = 0.08 ± 0.03) compared to T0 (0.17 ± 0.06) ($p < 0.05$). However, there were no statistical differences between the three toothpastes.

In contrast, different results were observed with regard to AbA. CSPS and HB (6.25 ± 13.48; 4.45 ± 3.06, respectively) were statistically different from T0 (20.49 ± 17.53) ($p < 0.05$). However, CHA (14.41 ± 19.66) was not different from T0, CSPS and HB ($p > 0.05$). Regarding patient appreciation, HB showed a higher VAS score (6.30 ± 2.08) than both CSPS and CHA (3.00 ± 2.08 and 3.05 ± 1.88, respectively). No adverse reactions were observed or reported during the trial. There was no report of discomfort related to the use of tested toothpastes.

4. Discussion

Both tested toothpastes, which contain desensitizing agents, CSPS and CHA, are commercially marketed to reduce not only DH but also gingival inflammation. Despite this, there has been no RCT to evaluate the anti-inflammatory activity of CSPS and CHA. Our double-blind three-treatment crossover trial, therefore, aimed to compare the anti-inflammatory activity of the two test toothpastes with HB, as the active control [23–26,31]. HB has been shown to exert antiplaque and anti-inflammatory activity. It contains naturally occurring substances, mainly sodium bicarbonate, which causes cell wall degradation and an enzymatic inhibition which in turn prevents bacteria from aggregating with



Gram-positive pioneer species and reduces bacterial multiplication [16,32]. The effectiveness of HB in reducing gingival inflammation and plaque accumulation has been well reported [33,34]. On the other hand, CSPS and CHA are toothpastes based on desensitizing agents. CSPS contains bioactive glass which, when exposed to body fluids, reacts and deposits hydroxycarbonate apatite on the exposed dentine; the deposited hydroxyapatite has a chemical structure similar to enamel and dentin [14,35,36]. CHA contains microcrystals with a low dissolution rate and releases ions such as Ca ions, phosphates and Zn ions, thus forming a biomimetic coating on the surface, which may remineralize the altered enamel surfaces and close dentinal tubules [15,37].

Since all three groups presented an FMPS value similar to that of the baseline, the tested toothpastes showed no evident antiplaque activity. One explanation of this result could be the so-called "sliding effect" [38]. It was hypothesized that the presence of toothpaste in sufficient amounts on the occlusal surface or the approximal sites could result in a reduction in the shear force of the toothbrush filaments in removing the adhered plaque [39]. Therefore, the present study supports the theory that the mechanical action provided by the toothbrush appears to be the main factor in the plaque removal process [19,38–41].

Although both CSPS and CHA showed the same reduction in FMBS compared to HB, only CSPS and HB (6.25 ± 13.48; 4.45 ± 3.06, respectively) showed a significant reduction in AbA compared to baseline (20.49 ± 17.53). Therefore, CSPS has been proven to be beneficial in reducing gingivitis. The present findings are only in partial agreement with the study of Aruna et al., which reported that use of a toothpaste containing calcium sodium phosphosilicate led to gingival bleeding reduction compared to a placebo toothpaste; however, a supra-gingival plaque reduction was also reported [12]. Moreover, the present results agree with Tai et al., which also demonstrated that a toothpaste containing calcium sodium phosphosilicate decreased gingival inflammation [42].

The possible AbA mechanism of action of CSPS could be explained by the antibacterial effect of high rates of ions released during the reaction in the oral cavity and the associated local changes in pH [43,44]. On the other hand, although there was a reduction in FMBS, CHA showed no different AbA from baseline. The aforementioned biomimetic coating formed by CHA on the tooth surface may be protective toward bacterial attacks [15,45]. Our finding disagrees with Hanning et al., who demonstrated antibacterial and antiadherent properties of CHA, although a mouth rinse with the same CHA composition was tested instead of a toothpaste [13].

In the present study, VAS was used to evaluate the subject's appreciation of the different toothpastes. HB was the most appreciated toothpaste (6.30 ± 2.08), while CSPS and CHA (3.00 ± 2.08 and 3.05 ± 1.88, respectively) showed similar yet lower VAS values. Toothpaste taste contributes to a fresh mouth experience which makes brushing an acceptable or even pleasant experience. In several studies, it was observed that brushing without the use of toothpaste was judged as unpleasant and discouraged patients from cleaning their teeth [38,46,47]. Although no article has compared the patient appreciation of the taste of the tested toothpastes, there is a study which has agreed with our findings of high patient appreciation of HB's taste [48]. Nevertheless, the results of this study should be interpreted with caution, as the sample size is small, and the sampling frame may not represent the general population.

5. Conclusions

In addition to their recognized desensitizing activity, both CSPS and CHA showed an anti-inflammatory effect comparable to HB. Moreover, CSPS showed an AbA higher than CHA and comparable to HB. Toothpastes containing CSPS, therefore, to a greater extent than CHA, may represent a clinically effective option for patients who suffer from both DH and gingival inflammation and therefore should be recommended by clinicians. Using one toothpaste instead of two different toothpastes is also a more practical option. Significantly, a toothpaste with a pleasant taste can improve oral hygiene compliance in the young and old. Therefore, an improvement of CSPS taste could be useful in making it more appreciated by patients and consequently, more frequently used. Further studies



are warranted to evaluate the efficacy and the antibiotic mechanism of action of 5% calcium sodium phosphosilicate bioactive glass toothpaste.

Author Contributions: R.M. contributed to the research protocol and wrote the present manuscript. S.S. contributed to the research protocol, collected the results and wrote the present manuscript. V.T. contributed to the analysis of all data and conducted the statistical advices. F.P. performed subject enrollment and contributed to the results collection and interpretation. M.M. conducted data interpretation and wrote the present manuscript. A.H. provided extensive english editing and substantially edited the introduction and discussion sections of the final draft. A.P. contributed to the research idea, made the critical revision of the paper and helped in the supervision of the project. G.O. also contributed to the research idea, supervised the project and cowrote the present manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Institutional funds of G.O. and A.P. The research fellowship of R.M. (first author) was partially granted by the Società Italiana di Odontostomatologia e Protesi Dentaria (SISOPD) and Curaprox, Italia Srl.

Acknowledgments: The toothpastes used for the study were kindly donated by Coswell S.p.A, Bologna, Italy and GlaxoSmithKline Consumer Healthcare, Brentford, United Kingdom. Moreover, the toothbrushes were provided by Curaden GmbH, Kriens, Switzerland.

Conflicts of Interest: The authors declare no conflict of interest.

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frontiers in Physiology

MINI REVIEW
published: 25 October 2018
doi: 10.3389/fphys.2018.01510



An Overview on Current Non-invasive Diagnostic Devices in Oral Oncology

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Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy, and despite advances in cancer therapies, the overall 5-year survival rate has remained below 50% over the past decades. OSCC is typically preceded by potentially malignant disorders (PMD), but distinguishing high-risk from low-risk PMD is challenging. In the last years, several diagnostic methods as light-based detection systems (LBDS) have been proposed to facilitate the detection of OSCC and PMD. Furthermore, the recent evolution of nanotechnology may provide new opportunities to detect PMD and OSCC at an early stage. Indeed, several preclinical studies showed the potential of nanotechnology to enhance diagnostic accuracy. For these reasons, it is fundamental to conduct studies to evaluate the efficacy of nanotechnology implementation in LBDS. The aim of this article is to review the current literature on LBDS and to provide a summary of the sensitivity and specificity of each technique, and possible future applications of nanotechnologies. The LBDS showed great potential for screening and monitoring oral lesions, but there are several factors that hinder an extensive use of these devices. These devices seem to be useful in assessing lesion margins that must be biopsied. However, to date, conventional oral examination, and tissue biopsy remain the gold standard for OSCC diagnosis. The use of nanotechnologies could be the next step in the evolution of LBDS, thus providing devices that can help clinicians to detect and better monitor oral lesions.

Keywords: light-based detection system, early diagnosis, OSCC, chemiluminescence, autofluorescence, nanotechnology

OPEN ACCESS

Edited by:
Gianpaolo Papaccio, Università degli Studi della Campania Luigi Vanvitelli, Italy

Reviewed by:
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Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *30 July 2018*
Accepted: *08 October 2018*
Published: *25 October 2018*

Citation:
Mascitti M, Orsini G, Tosco V, Monterubbianesi R, Balercia A, Putignano A, Procaccini M and Santarelli A (2018) An Overview on Current Non-invasive Diagnostic Devices in Oral Oncology. Front. Physiol. 9:1510. doi: 10.3389/fphys.2018.01510

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy and the sixth most common tumour worldwide (Warnakulasuriya, 2009). Despite advances in therapies, the overall 5-year survival rate has remained unchanged during the past decades, mainly due to delayed diagnosis (Gomez et al., 2009). OSCC is typically preceded by potentially malignant disorders (PMD), a group of clinically suspicious lesions. Although the majority of PMD do not progress to OSCC, distinguishing high-risk PMD from low-risk PMD is challenging for dental practitioners (Yang et al., 2018). Furthermore, patients treated for OSCC are at risk of developing recurrences and secondary primary tumours, due to field cancerization and/or incomplete surgery (Day and Blot, 1992). Currently, conventional oral examination (COE), consisting in visual and tactile assessment of accessible oral structures, followed by tissue biopsy still constitutes the gold



standard for diagnosis of PMD and OSCC. However, there are some limitations of this procedure, such as sampling bias that can lead to underdiagnosis or misdiagnosis, particularly in multifocal lesions (Yang et al., 2018).

The possibility of making an early diagnosis is crucial for reducing high mortality rate and morbidity of OSCC patients. In the last years, several light-based detection systems (LBDS), based on optical properties of biological tissues, have emerged with claims of enhancing oral mucosal examinations and facilitating the detection of PMD and OSCC.

Furthermore, the recent evolution of nanotechnology may provide new opportunities to detect PMD and OSCC at an early stage (El-Sayed et al., 2005). Several preclinical studies showed the potential to enhance diagnostic accuracy of optical diagnostic technologies (e.g., Raman spectroscopy) or imaging techniques (e.g., Magnetic resonance imaging) (Chen et al., 2018). Among the latter techniques, reflectance confocal microscopy seems to improve the evaluation of oral lesions, by detecting backscattered light from illuminated tissue, producing high resolution tissue map. However, several technological limitations need to be resolved to validate diagnostic accuracy (Lucchese et al., 2016). LBDS showed several advantages compared to the aforementioned approaches, such as low cost and ease of use. For these reasons, it is fundamental to conduct studies to evaluate the efficacy of nanotechnology implementation in LBDS.

The aim of this article is to review the literature on LBDS currently on the market (**Tables 1**, 2), providing clinicians with a better understanding of their advantages and limits, and possible future applications of nanotechnologies.

ViziLite®

ViziLite® (Zila Pharmaceuticals, Phoenix, AZ, United States) is a chemiluminescence-based detection device designed to facilitate the early identification of PMD and OSCC. In 2002 ViziLite® became the first device approved by FDA for this purpose (Oh and Laskin, 2007). This is a disposable capsule formed by an outer shell of flexible plastic containing acetyl salicylic acid and an inner glass vial containing hydrogen peroxide. To activate it, the capsule is bent to break the inner glass vial, triggering the reaction of the chemicals contained in the two compartments. Consequently, a bluish-white light (430–580 nm) is produced,

TABLE 1 | Published studies on VELscope® for clinical detection of oral lesions.

Author and year	Patients	Type of lesion	Sens	Spec	PPV	NPV
Poh et al., 2006	20	OSCC	95%	–	100%	–
Lane et al., 2006	44	PMD, OSCC	98%	100%	100%	86%
Roblyer et al., 2009	65	OL	95.9%	96.2%	–	–
Jayaprakash et al., 2009	60	OL	72%	50%	76%	46%
Mehrotra et al., 2010	156	OL	50%	38.9%	6.4%	90.3%
Koch et al., 2011	78	PMD, OSCC	94%	16%	45%	77%
Paderni et al., 2011	175	PMD, OSCC	80.7%	97.5%	93.9%	91.3%
Awan et al., 2011a	126	PMD	84.1%	15.3%	37.8%	61.1%
Scheer et al., 2011	64	OSCC	100%	80.8%	54.5%	100%
Marzouki et al., 2012	85	OL	92%	77%	–	–
McNamara et al., 2012	130	OL	66.7%	6.0%	4.1%	75%
Farah et al., 2012	112	PMD	30%	63%	19%	75%
Rana et al., 2012	123	PMD	100%	74%	16.7%	100%
Hanken et al., 2013	60	PMD	97.9%	33.3%	85.5%	80%
Sawan and Mashlah, 2015	71	OL	100%	74.1%	46.4%	100%
Kaur and Jacobs, 2015	130	OL	67%	62%	29.8%	89%
Jane-Salas et al., 2015	60	OL	40%	80%	62.5%	66.7%
Elvers et al., 2015	20	PMD	–	–	–	–
Ayoub et al., 2015	30	Screening	–	–	–	–
Ohnishi et al., 2016	20	OSCC	91%	100%	100%	58.3%
Scheer et al., 2016	41	OSCC	33.3%	88.6%	33.3%	88.6%
Yamamoto et al., 2017	62	PMD, OSCC	85.9%	26.7%	83.3%	30.8%
Ganga et al., 2017	200	OL	76%	66.3%	24.4%	95.1%
Burian et al., 2017	90	OSCC	–	–	–	–
Huang et al., 2017	140	PMD, OSCC	98.3%	77.6%	91.7%	93.8%
Amirchaghmaghi et al., 2018	21	PMD, OSCC	90%	15%	40%	71%
Farah et al., 2018	11	PMD	–	–	–	–
Canjau et al., 2018	18	OL	94.4%	100%	100%	50%

OL, oral lesions; PMD, potentially malignant disorders; OSCC, oral squamous cell carcinoma; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value. Where possible, missing data were recalculated.



TABLE 2 | Published studies on light-based detection systems other than VELscope® for clinical detection of oral lesions.

Author and year	Device	Patients	Type of lesion	Sens	Spec	PPV	NPV
Huber et al., 2004	ViziLite	150	PMD	–	–	–	–
Ram and Siar, 2005	ViziLite	40	PMD, OSCC	100%	14.3%	80%	100%
Epstein et al., 2006	ViziLite	134	PMD	–	–	–	–
Kerr et al., 2006	ViziLite	501	OL	–	–	–	–
Farah and McCullough, 2007	ViziLite	55	OL	100%	0%	18.2%	–
Oh and Laskin, 2007	ViziLite	100	Screening	–	–	–	–
Epstein et al., 2008	ViziLite	84	PMD	100%	55%	37%	100%
Mehrotra et al., 2010	ViziLite	102	OL	0%	75.5%	0%	94.8%
Awan et al., 2011b	ViziLite	126	OL	87%	24%	15%	92%
Mojsa et al., 2012	ViziLite	30	PMD	57.6%	37.5%	79.2%	17.6%
Rajmohan et al., 2012	ViziLite	30	PMD, OSCC	85%	100%	100%	76.9%
Ujaoney et al., 2012	ViziLite	44	PMD	59%	78%	–	–
Vashisht et al., 2014	ViziLite	60	PMD, OSCC	95.5%	84.6%	91.3%	91.7%
Kammerer et al., 2015	ViziLite	44	PMD	100%	30%	26%	100%
Chaudhry et al., 2016	ViziLite	100	PMD	84.8%	41.2%	58.3%	70%
Sweeny et al., 2011	Identafi	88	PMD (white)	50%	98%	50%	98%
			(violet)	50%	81%	11%	97%
			(green)	0%	86%	0%	95%
Lane et al., 2012	Identafi	124	PMD	82%	87%	–	–
Messadi et al., 2014	Identafi	21	OL	–	–	–	–
Lalla et al., 2015	Identafi	342	screening	–	–	–	–
Lalla et al., 2016	Identafi	88	OL (white)	100%	100%	100%	100%
			(Violet)	27.5%	96.3%	61.1%	86.4%
			(Green)	40%	71.7%	22.9%	85.1%
McIntosh et al., 2009	Microlux/DL	50	PMD	77.8%	70.7%	36.8%	93.5%
Ibrahim et al., 2014	Microlux/DL	599	Screening	100%	32.4%	17.9%	100%
Moro et al., 2010	GOCCLES	32	PMD, OSCC	100%	93%	92%	100%
Moro et al., 2015	GOCCLES	61	PMD	96.9%	3.1%	50%	50%

OL, oral lesions; PMD, potentially malignant disorders; OSCC, oral squamous cell carcinoma; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value. Where possible, missing data were recalculated.

lasting for 10 min (Liu et al., 2016). A modified version (ViziLite® PLUS) consists of a combination of chemiluminescence and toluidine blue (TB) marking system, an acidophilic dye that selectively stains acidic substances such as DNA. Furthermore, an accessory eyewear has been developed, to allow better isolation of chemiluminescent light (Sambandham et al., 2013). Its clinical use requires a 1-min rinse of 1% acetic acid solution, to desiccate oral tissues, followed by oral examination with 430–580 nm wavelength light. The altered epithelial cells, due to higher nuclear/cytoplasmic ratio, reflect the light and cause the appearance of an "aceto-white" lesion, whereas normal cells appear blue (Nagi et al., 2016).

The first studies regarding ViziLite®, published in 2004–2007, were conducted on subjects with different clinical conditions, ranging from normal mucosa to diagnosed OSCC, with the aim to explore the diagnostic utility of chemiluminescence-based strategies (**Table 2**). In the first reported study, ViziLite® identified a subclinical lesion, suggesting its utility in identifying occult epithelial abnormalities (Huber et al., 2004). In a small cohort of patients with oral lesions, ViziLite® appears to be a better diagnostic tool than TB in detection of OSCC and PMD (Ram and Siar, 2005). Another study highlights the ability of ViziLite® to show brighter and better demarcated lesions than using incandescent light, aiming to enhance the identification of lesions that could be biopsied (Epstein et al., 2006). Unfortunately, these results have not been confirmed, which failed to demonstrate significant improvement in identification and evaluation of oral lesions (Farah and McCullough, 2007; Oh and Laskin, 2007). Interestingly, a cross-sectional study compared ViziLite® and VELscope® to evaluate their clinical utility in diagnosing oral lesions, but the authors failed to demonstrate any superiority to COE (Mehrotra et al., 2010).

For this reason, a new version of this device has been developed (ViziLite® PLUS), aiming to improve the diagnostic power of TB marking system. First results were encouraging, showing that TB reduced the number of false positive cases leaving the false negative rate unchanged (Epstein et al., 2008). On the contrary, ViziLite® PLUS does not seems to be useful to detect malignancies in patients with clearly visible lesions (Mojsa et al., 2012). In fact, some authors described the better diagnostic accuracy of ViziLite® with respect to TB staining alone (Rajmohan et al., 2012; Vashisht et al., 2014), justifying the combined use of these two techniques.



Recently, the results of a clinical study suggested that, although the adjunct of TB to ViziLite® reduced the false positive cases without increasing the number of false negatives, there are little benefits in using this device in general dental practise (Chaudhry et al., 2016).

In conclusion, despite the fact that ViziLite® facilitates the identification of hyperkeratotic areas and may increase the visibility of mucosal lesions, the main limitation is currently the high proportion of false positive and false negative tests, regarding the identification of dysplastic areas rather than hyperkeratosis (Chhabra et al., 2015).

VELscope®

VELscope® (LED Medical Diagnostics, White Rock, BC, Canada) is a hand-held non-magnifying device for direct visualisation of oral mucosa autofluorescence that became commercially available after FDA approval in 2006 (Ayoub et al., 2015). No need of technical measures, such as the use of dimmed light, pre-rinse or lesion-marking solutions, make VELscope® easy to use. It uses a 120 W arc-lamp and a series of philtres and reflectors optimised for producing 400–460 nm wavelength light. The light emitted reaches oral mucosa and excites endogenous autofluorescence substances, called fluorophores (Yamamoto et al., 2017). Preliminary studies, regarding small groups of patients, gave encouraging results (**Table 1**). In the first reported study, 44 patients with confirmed oral dysplasia or OSCC were evaluated with both COE and VELscope®. The results showed that the device can differentiate PMD and OSCC from normal oral mucosa, with high sensitivity and specificity levels (Lane et al., 2006). These results were confirmed in a small OSCC cohort study, in which the use of autofluorescence-guided examination was able to identify subclinical high-risk fields with cancerous changes (Poh et al., 2006). In a study conducted on 60 patients using a semi-quantitative grading system for autofluorescence, VELscope® demonstrate good sensitivity and a better ability to recognise high-grade lesions than COE (Jayaprakash et al., 2009). Another study evaluated 65 subjects with VELscope®, using a specific algorithm based on the ratio of red-to-green fluorescence. The authors found that 405 nm wavelength light was able to discriminate neoplastic and non-neoplastic tissue with high sensitivity and specificity (Roblyer et al., 2009).

In a cross-sectional study, 175 patients with at least one clinical lesion were evaluated using VELscope®. However, despite the good results, the authors warned that this device could lead to overdiagnosis if used by non-specialists (Paderni et al., 2011). In fact, in the following years several studies on patients with PMD or OSCC reported low specificity values, highlighting this as the primary limitation of VELscope® (Awan et al., 2011a; Koch et al., 2011; Scheer et al., 2011). For these reason, other authors concluded that VELscope® examination alone does not provide significant diagnostic benefit beyond COE in screening for PMD and OSCC, also due to interobserver variability (Farah et al., 2012; McNamara et al., 2012). These results were confirmed by a study on 200 patients, limiting the utility of autofluorescence for OSCC screening (Ganga et al., 2017).

One effort to overcome these shortcomings consists of adding the VELscope® exam to the COE. Indeed, as reported by several authors, the combination of COE and VELscope® examination in patients with oral lesions could provide a significative diagnostic yield (Marzouki et al., 2012; Rana et al., 2012; Hanken et al., 2013). However, these results must be interpreted carefully due to the different inclusion and exclusion criteria used to select the patient cohorts, which can influence both sensitivity and specificity.

Other studies focused on combining VELscope® and other diagnostic tests, aiming to find out better approaches to improve detection of PMD and OSCC. For example, the combination approaches of tissue autofluorescence and salivary protoporphyrin IX levels seems to be effective to distinguish between normal mucosa and high-risk lesions (Kaur and Jacobs, 2015). The use of quantitative analysis of autofluorescence were developed to solve the problem of interobserver variability. Novel methods such as quadratic discriminant analysis or luminance ratio were promising, showing a strong concordance with histopathological diagnosis (Huang et al., 2017; Yamamoto et al., 2017). Recently, a retrospective study based on oral photograph was conducted to find colour distribution patterns related to neoplastic lesions. The fluorescence analysis showed differences in the red-to-green ratios of neoplastic areas, suggesting its clinical utility to detect early OSCC (Burian et al., 2017).

Recently, tissue autofluorescence was used to investigate biological aspects of oral carcinogenesis. In the first *in vitro* study, VELscope® was used to investigate the autofluorescence in a rat tongue carcinogenesis model. The results showed significant changes in autofluorescence pattern during progression to dysplasia and carcinoma (Ohnishi et al., 2016). In another study, RNA sequencing technique was used to identify molecular differences related to autofluorescence patterns. Results were encouraging, demonstrating that the autofluorescence-based excision was successful in achieving a clear molecular margin when excising PMD (Farah et al., 2018). These results confirmed those previously reported in literature, in which VELscope® demonstrated that the actual sizes of some lesions are significantly larger than they look clinically (Elvers et al., 2015).

In conclusion, several criticisms have been made about VELscope®, mainly focused to the limited capacity to extend the use of this device in general dental practise. Future research directions are aimed at improving the specificity of this device, allowing wider clinical use of VELscope® in routine general practise (Bhatia et al., 2013).

Identafi®

Identafi® (StarDental - DentalEZ, Lancaster, PA, United States) is a probe-like device designed for multispectral screening of PMD, approved by FDA in 2009 as oral screening device (Vigneswaran et al., 2009). Identafi® has three light sources of different wavelengths: white, violet (405 nm), and green-amber (545 nm) lights, that can be sequentially used in oral examination. While white light provides classical visualisation of oral mucosa, violet light excites endogen fluorophores, enabling the assessment of mucosa autofluorescence, like VELscope®. Green-amber

light, through the reflectance spectroscopy, excites haemoglobin molecules in the blood, with the aim to visualise the vasculature (Messadi et al., 2014). A mirror is attached to the probe to help visualise relatively obscure areas in oral cavity.

The first clinical trial with Identafi® was conducted on 88 patients who were treated previously for OSCC (**Table 2**). Screening results with white, violet, and green lights were compared to each other, showing limited benefits of tissue reflectance and autofluorescence in detecting high-risk lesions (Sweeny et al., 2011). In 2012, was reported a case series of PMD patients with the aim to evaluate the efficacy of Identafi®. Although the results are not clearly described, this device seems to be helpful in identifying characteristics not otherwise visible to the COE (Lane et al., 2012).

In a pilot study, Identafi® was used to evaluate tissue vascularity of PMD and to compare with the histological grading of the lesions using a vascular marker (CD34). The results found a correlation between tissue reflectance and histological assessment of vascular structure, in both OSCC and non-cancerous lesions (Messadi et al., 2014).

Two studies on the effectiveness of Identafi® were conducted on Australian population. In the first one, 342 urban Indigenous community members were screened for oral lesions using reflectance spectroscopy and autofluorescence imaging. Identafi® improved the visibility of oral cavity lesions and was capable to find new lesions not seen during COE, although the prevalence of oral pigmentation in this community could hamper the use of autofluorescence screening systems (Lalla et al., 2015). In the second study, 88 patients were evaluated with Identafi®, showing good specificity, negative predictive value, and accuracy (Lalla et al., 2016).

Taken together, the use of Identafi provide the clinician with more data than COE. Unfortunately, the results interpretation requires high level of experience and clinical training in oral pathology, suggesting that its usage should be limited to reference centres for oral pathology (Lalla et al., 2016).

OTHER DEVICES

Microlux/DL™ (AdDent Inc., Danbury, CT, United States) is a chemiluminescence-based device which became commercially available after FDA approval in 2005. This device has a diffused blue-white LED light source and a fibre optic light guide (McIntosh et al., 2009). It uses the same principles of ViziLite®: after 1-min rinse with 1% acetic acid, oral examination is performed with 460–555 nm wavelength light. Altered epithelial cells cause the appearance of "aceto-white" lesions, and LED light source makes the lesion more easily recognisable. Furthermore, the use of TB can be used in conjunction with Microlux/DL™, to enhance the visualisation of dysplastic areas (Ibrahim et al., 2014). In 2009 was conducted a study on 50 patients with oral white lesions to evaluate the efficacy of Microlux/DL™. The results showed that this device can enhance visualisation of oral mucosa, but no clinical improvement was observed, due to poor ability to distinguish between benign and malignant lesions (McIntosh et al., 2009). Another trial that evaluated the effectiveness of Microlux/DL™ was carried out in 2014. 599 patients were examined with COE and Microlux/DL™ with and without TB, showing high sensitivity but low specificity, indicating that this device is not effective to distinguish between benign and malignant lesions, although seems to be a promising screening test for oral lesions (Ibrahim et al., 2014).

GOCCLES® is a medical device (Pierrel S.p.A, Italy) approved by FDA in 2015. This is a low cost and easy-to-use device consisting in a pair of glasses equipped with special optical philtres that allows autofluorescence detection. Indeed, GOCCLES® was created to provide an easy and low cost mean of identification of autofluorescence abnormalities in oral cavity with the use of any dental curing light (Moro et al., 2015). In 2010 was reported the first study on GOCCLES® in a small cohort of selected patients, showing high sensitivity and specificity (Moro et al., 2010). Five years later, a non-randomised multicentre trial was conducted on patients at risk for OSCC, suggesting the need for further researches to define the diagnostic performance of this device (Moro et al., 2015). Indeed, despite the low cost of GOCCLES® could encourage more careful examinations, its main limitation seems to be the interobserver variability, that could be overcome by proper training.

In recent years, other instruments have been developed and commercialised for facilitate the early identification of oral lesions. Their operating principle is equivalent to the devices described above, using either autofluorescence or chemiluminescence detection. However, their clinical effectiveness is currently hampered by the lack of published studies. For these reasons, they will only be mentioned briefly here. Bio/Screen® (AdDent Inc., Danbury, CT, United States), an instrument with five violet (390–430 nm) high-power LED, designed to enhance the visualisation of mucosal abnormalities through the use of tissue autofluorescence (Kahn et al., 2018).

Orascoptic DK™ system (Orascoptic, Middleton, WI, United States) is another chemiluminescence-based device, designed to improve the visualisation of oral lesions through the use of blue-white LED light and oral rinse of 1% acetic acid solution (Patton et al., 2008).

Sapphire® Plus LD (DenMat Holdings, Lompoc, CA, United States), DentLight DOE™ Oral Exam System (DentLight, Richardson, TX, United States), and OralID™ 2.0 (Forward Science Technologies, Stafford, TX, United States) are other tissue autofluorescence-based devices developed in order to detect oral lesions (Kahn et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

The diagnostic techniques presented here showed great potential for screening and monitoring oral lesions (Liu et al., 2016). Unfortunately, to date several factors hinder an extensive use of these devices: (1) data do not demonstrate clear superiority of these methods compared to COE; (2) there remains the need for well-designed multicentre prospective studies; (3) these devices

exhibit a not-negligible interobserver variability, limiting their use to clinicians with significant experience in oral pathology (Patton et al., 2008; Carreras-Torras and Gay-Escoda, 2015).

However, the current evidence suggests that these devices: (1) seem to be useful in assessing lesion margins that must be biopsied and, therefore, may be useful in surgical management; (2) can be used to investigate biological aspects of oral carcinogenesis, leading to more accurate methods for interpreting data from LBDS; (3) can be enhanced with new approaches used to analyse optical imaging data, with the aim to quantify the results obtained; (4) lowering the costs of these devices could indirectly lead to greater attention for oral lesions among both patients and general dental practitioners, allowing in turn to promote a culture of oral cancer prevention (Carreras-Torras and Gay-Escoda, 2015; Moro et al., 2015); (5) finally, the possibility of implementing LBDS through the use of tissue-marking dyes can in principle allows to develop strategies for the use of nanoparticles. Indeed, nanoparticles can provide molecular targeted imaging, with higher image contrast and resolution. For example, a promising nanotechnology in oral diagnostic research is the quantum dots, consisting in nanometre-sized semiconductor crystals (Walling et al., 2009). The biophysical characteristics of these particles confer several advantages over conventional dyes and fluorescent proteins. The possibility to link the quantum dots to molecules with the ability to target cancer cells make them ideal for diagnostic applications in detecting PMD and OSCC (Chen et al., 2018). Therefore, the use of nanotechnologies could be the next step in the evolution of LBDS, providing devices that can help clinicians to detect and better monitor oral lesions.

AUTHOR CONTRIBUTIONS

MM, AS, and MP conceived the literature review. MM and VT described the VELscope® system. AS and RM described the ViziLite® system. AB and GO described the Identafi® system. AP, MP, and GO wrote the concluding remarks. All authors discussed and approved the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2. Nanotechnological Application in Aesthetic Dentistry

Aesthetic dentistry is not considered a real dentistry discipline by itself, although it represents one of the goals of dental treatment interventions due to its functional and biological considerations, spanning all specialty areas, from preventive and restorative dentistry to prosthodontics, orthodontics, periodontics, as well as oral and maxillofacial surgery (9). Furthermore, the need of patients to improve their smile implies greater research and ability for the clinician to imitate the tooth nature as faithfully as possible, understanding the layout, positions, proportions, shapes, color and morphologies of natural teeth. Strong scientific evidence was observed regarding the appearance of a person's face (21,146) and the teeth (127), highlighting a profound impact on the perception and judgment by others. Indeed, aesthetically attractive teeth are associated with kindness, popularity, intelligence, and high social status (158). Moreover, the level of pleasure with one's own smile attractiveness is directly correlated with self- perception and evident psychological traits, ultimately affecting overall well-being and health (136,169).

In spite of the widely evidence of patient's demands for achieving an attractive smile, the actual need for aesthetic dental treatments has always been discussed controversially, due to ethical concerns (107). Therefore, the understanding and realizing the patient's aesthetic needs play a crucial role for the clinician, and sometimes an improper and overtreatment lead to severe detrimental consequences on the attractiveness and well-being of the patient.

Consequently, the application of nanotechnology to aesthetic dentistry provides reliable and effective procedures for the best final accomplishment with the patient satisfaction. Nowadays, the use of a complete digital process for the diagnosis, design, and manufacturing of the complete maxillary arch restorations on teeth and implants, resulted in increased efficiency,



accuracy, and overall effectiveness of patient's treatments. New digital tools have revolutionized clinical and laboratory key steps, from the first visit to final treatment, facilitating the communication with the patient, as well as the treatment planning (63). The number of new technologies and the amount of new equipment used today to acquire patients data, the software to manipulate these data, and the machines to manufacture the devices are continuously increasing, as are the challenges of integrating these systems into a feasible, realistic, and practical workflow (30). Nowadays, the digital workflows represent even more the routine rather than the exception in a dental practice.

Currently, several smile designs are available for clinicians, assuring a useful tool for team communication and patient motivation. All of these software rely on the input and preference of the clinician to design the shape and alignment of the future smile (27). Once the photos of the patient are uploaded, the digital smile software searches and proposes natural shapes of teeth and alignment, creating a personalized new smile design for each specific patient (64). Furthermore, several intraoral scanners have been progressively used to make digital scans of dental arches, presenting also an innovative and interesting shade-taking function to identify the color of a tooth and to reproduce it in a dental restoration (151). Indeed, the color analysis and detection play a fundamental step that has always represented the greatest challenge in aesthetic dentistry.

2.1 Tooth color

The color of the teeth depends on intrinsic and extrinsic colorations. Intrinsic color refers to light scattering and absorption of the enamel and dentin, while extrinsic color is determined by the absorption of materials onto the tooth surface (13). They are a result of optical properties related to transmission, absorption, scattering, and light reflection. The dentin gives color to



the tooth, while enamel scatters the external light (13). The perception of color is influenced by the light source, the object being viewed, and the observer (84). It is therefore difficult to communicate color with others, and several color scales have been developed for that purpose.

Color can be described both qualitatively and quantitatively. The Munsell system is qualitative which the color was positioned in a space and described according to three parameters or "dimensions": value, hue, and chroma (77).

Value indicates the degree of lightness or darkness, the amount of white or gray; it can assume values ranging from 0 to 10. At 0 there is absolute black while at 10 pure white. Between these two extremes are the shades of gray, defined by Munsell himself as achromatic colors.

Hue defines the proper color of the tooth; the quality through which we distinguish one color from another, such as red from yellow. In Munsell's space the main colors are red, yellow, green, blue and purple and are placed in a circle at regular intervals around a vertical axis.

Chroma is the amount of saturation or intensity of the hue. Represents the quality thanks to which we distinguish a strong color from a weak one; the intensity of the color.

“La Commission Internationale de l’Eclairage” (CIE) developed a quantitative system that describes color as the product of blending three color coordinates, which represent how the human visual system responds to a given color: L*, a*, and b* (Figure 1)(83).

L* represents lightness ranging from black to white (0=pure black, 100=pure white),

a* identifies redness ranging from red to green (+a=redness, –a=greenness),

b* defines yellowness ranging from yellow to blue (+b=yellowness, –b=blueness).



Figure 1. The three values defined by CIELab* system.

The CIELab* system, introduced in 1976 and 1978 (181), was first to express color by numbers and calculate differences in relation to visual perception (84).

The calculation and measurement of the color difference (ΔE) between the 2 objects can be achieved by using the CIELab* system. Under experimental conditions, when ΔE >1, the color difference can be seen by the human eye (96), but the clinically visible ΔE for a tooth has been reported to be when ΔE >3.7 (82). When the tooth color is determined with shade guides, several shade tabs could be visually acceptable, since the tabs and the tooth must have ΔE less than 3.7. To identify the color of a tooth and to replicate it in a dental restoration, commercial shade guides have been used. One of the most popular shade guides, the VitaClassical (VC) (Vita Zahnfabrik), is based on the parameters of natural teeth color, above mentioned. Several shade guides competing (Noritake; Kuraray Noritake Dental Inc, Chromascop; Ivoclar Vivadent AG, Bioform; Dentsply Sirona) are also based on the same



principle (7); whereas, Vita 3D-Master (Vita Zahnfabrik) shade guide is systematically arranged on the CIELCh color scale (based on L*, chroma and hue) and has been reported to be more reliable (1,130). In addition, a custom shade guide was introduce to obtain a reliable system designed to produce self-made standardized "tooth-shaped" shade guide specimens, using the restorative material itself (135).

Therefore, color-measuring instruments and systems have become increasingly popular, especially in dental research. Digital cameras, spectrophotometers, and colorimeters have been applied to detect the color assuring to be more reliable than the visual method (24), over to determine the translucency of the tooth, which is the second leading factor, after color, for matching a restoration to adjacent teeth (104). Despite spectrophotometers are among the most accurate devices for color tooth detention (24), recent works have claimed that, in terms of accuracy, some complete-tooth measurement colorimeters provide results similar to spectrophotometers ones (12). In addition, the recent application of new digital imaging systems for color measurements are comparable to spectrophotometry, providing additional information and measuring appearance attributes beyond intrinsic color (84).

In conclusion, the clinicians desire to reproduce natural optical teeth features and to meet the aesthetic requirements of patients, combine with the nanotechnological application, has led to the development of new color measuring instruments and new software, that represent an excellent opportunity to make these procedures simple and affordable for everyone.

2.2 Dental bleaching

One of the principal challenges of clinicians in performing aesthetically accepted dental treatments is undoubtedly the dental bleaching. In the last decades, patients' demand for tooth



whitening procedures has considerably risen, thus becoming an important and attractive topic in dentistry (37).

The bleaching procedures consist on the application of oxidizing agents, which directly break the double bonds of chromophores, correcting and reducing intrinsic discolorations and/or external discoloration resistant to cleaning (35,81,179). This process produces smaller molecules that absorb light with a shorter wavelength than visible light (such as ultraviolet light), and hence the teeth no longer appear to be colored (33,34).

Despite its popularity and well-recognized acceptance, there are potential severe side effects associated with the use of chemical oxidants, such as gingival irritation, modifications in surface morphology and tooth hypersensitivity (138,148). Some researchers have evaluated the effects of aggressive bleaching treatments on tooth structure, observing changes in the surface integrity and on the microstructure of enamel crystals, and a higher susceptibility to demineralization (20,159,167). Regarding the common in-office products available on the market, the different concentrations of peroxide range from 5 to 40% of hydrogen peroxide (HP) or carbamide peroxide (CP) (62). It is believed that HP penetrates enamel and dentin and, due to its chemical instability, it is decomposed into different active oxygen species under specific temperature, pH and light conditions (100); these free radicals may oxidize the conjugated chain of the chromophores (100), and, furthermore, the oxidation could promote morphological changes of the enamel (90). On the other hand, CP dissociates approximately for a third of former concentration into HP and urea, which further breaks down into water and ammonia (31). Furthermore, the risk of adverse effects rises with increasing HP concentration (137). Fearon compared a number of studies of different whitening procedures and concluded that changes in the tooth surface structure and increased tooth sensitivity can occur, especially if highly concentrated HP solutions are applied (49).



The clinical application of nHA represents the last frontier to prevent the bleaching side effects, within different carriers. Hydroxyapatite is a calcium phosphate compound with the molecular formula of Ca_{10} (PO_4) OH_2 and a calcium to phosphorus ratio of 1:67. There are several forms of calcium phosphate in nature, the HA representing the most stable one (11). It can be considered as a revolutionary material due to its remarkable remineralizing effects on enamel initial lesions, thus performing also a protective action against caries and dental erosion (74). The nHA presents a strong affinity with demineralized surfaces due to its ability to bind to pores created by acid attacks. Indeed, after adhering, nHA multiplies and organizes into microclusters to form an uniform apatite layer, that may completely overlap interprismatic and prismatic enamel (163). According to these findings, in order to prevent hypersensitivity after bleaching, which can occur in a 70% of bleached patients, whitening gel can be enriched with HA in its nano form (99).

Despite this current new whitening strategy, its color change effectiveness it still uncertain. Several studies have evaluated the L*, a*, and b* coordinates of the CIELab* system (ΔE), in order to detect color alteration capability of bleaching techniques (84,98). Considering these parameters, several studies have claimed that the L* values spectrophotometric measurements, increased significantly in after bleaching, regardless of the presence or absence of nHA. Therefore, 6% HP gels combined with nHA, are effective for the whitening treatment (38,88,117). Regarding the b* parameter, which represent blue-yellow contrast, its decreased value at the end of treatment indicates a general improvement in the observation of the whites of the teeth (165). This result is confirmed by studies which indicate that the color change from yellow to blue represents significant evidence of whitening and that a decrease in the value of the b* parameter is a crucial factor for the recognition of teeth whitening by patients (8,80). Indeed, a change or shift to blue creates a tooth appearance that visually looks whiter, changing the sharp color towards white. This whitening mechanism is based on many previous



studies performed which have shown that the blue shift of the yellow-blue (b*) axis is of greater importance in observing whiter teeth than other axes, such as L* or a* (58,59,93). Furthermore, based on this optical theory of b* value, a blue pigment nanoparticle, called “covarin blue”, was introduced in the whitening gels to increase the color change of the teeth from yellow to blue. Several clinical and in vitro studies have confirmed that this increase in bluish color and decrease in yellow color makes teeth appear whiter immediately after teeth whitening (128,157,171).

Indeed, the enamel structure plays an important role in the wellbeing of dental hard tissues, and hence, the investigation of hardness and morphological changes of enamel surface become determinant even more. The surface hardness of enamel refers to dental resistance against the process of decay. Since the surface layer of enamel has a decisive role in demineralization process, evaluation of changes in this area has great importance. The Vickers Hardness test (VH) is widely utilized to analyze microhardness enamel surface (29,118,174). SEM analysis allows to obtain a qualitative examination of the enamel morphology changes at high magnification (177).

According to recent clinical studies, the current strategies to avoid the discomfort and damages associated with bleaching treatments are not totally effective, and they are not being optimized yet (42,148,168). Therefore, it is necessary to investigate the real potential of nHA application on bleaching products, providing an optimum whitening effect without any damage.

In this light, the study realized in collaboration with the visiting Professor, Dr. Mutlu Özcan, Division of Dental Biomaterials, Clinic for Reconstructive Dentistry, University of Zurich, (Switzerland), regarding the behavior of different treatment with HP bleaching agents on enamel structure, has obtained very promising results [Paper VI]. The tester groups, consisting on different HP concentration of gels containing nHA, and the other one, based on a 6 %CP gel, were compared with an untreated enamel surface (Control), and evaluated by SEM, Attenuated



Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Energy Dispersive X-ray Spectroscopy (EDS). The results obtained prove that the application of a HP concentration up to 12%, associated with nHA, does not modify the morphological and chemical composition of the enamel surface and maintains its crystallinity.

In conclusion, nanotechnology is again fundamental to improve the quality of whitening treatments for both the clinician and the patient. Furthermore, the nHA plays a crucial role in repairing the altered enamel morphology, thanks to its major advantages as the analogy to the mineral structure of teeth, its bioactivity and biocompatibility. Therefore, bleaching agents containing nHA may represent the key to address the numerous challenges regarding dental whitening procedures.



Paper VI

VI. Orilisi G, Tosco V, Monterubbianesi R, Notarstefano V, Özcan M, Putignano A, Orsini G (2021) ATR-FTIR, EDS and SEM evaluations of enamel structure after treatment with hydrogen peroxide bleaching agents loaded with nano-hydroxyapatite particles. PeerJ 9:e10606 DOI 10.7717/peerj.10606



PeerJ

ATR-FTIR, EDS and SEM evaluations of enamel structure after treatment with hydrogen peroxide bleaching agents loaded with nano-hydroxyapatite particles

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ABSTRACT

Background: Tooth whitening is one of the most requested dental treatments, but it still presents some side effects. Indeed, the bleaching agent can generate patients' discomfort and dental hard tissue damages, not achieving an efficient and long-lasting treatment with optimum whitening effect. To overcome these limitations, the bleaching agents containing nano-hydroxyapatite can represent a reliable solution to avoid these detrimental effects.

Methods: In this study, human third molars were treated with commercial bleaching agents, containing nano-hydroxyapatite (nHA) and 6% (at-home treatment), 12% and 18% (in-office treatments) of hydrogen peroxide (HP), named respectively G1, G2 and G3. The results were evaluated descriptively and analytically using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray spectroscopy (EDS), comparing the treated groups with a commercial gel containing 10% carbamide peroxide (CONV) and with a non-treated control group (CTRL).

Results: ATR-FTIR analysis revealed a similar composition in carbonates and phosphates for CTRL, G1 and G2 groups, which showed no significant differences in their spectral profiles; an increased amount of organic matter was found in G3, while CONV displayed an intermediate behavior. SEM analysis did not highlight significant changes in the enamel microstructure of G1 and CONV when compared to CTRL; the pattern observed in G2 presented a slight increase of enamel irregularities, while G3 displayed a partial removal of the aprismatic layer and microporosities. No evident effects due to nHA were observed in the structure of the hydroxyapatite component of G1, G2 and CONV, if compared to CTRL, while G3 showed a slight loss of crystallinity. In all groups, EDS identified slight changes in the concentration of chemical elements O and Ca, while the Ca/P ratio was similar when compared to CTRL.

Submitted 16 July 2020
Accepted 28 November 2020
Published 29 January 2021

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Academic editor
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Additional Information and Declarations can be found on page 18

DOI **10.7717/peerj.10606**

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How to cite this article Orilisi G, Tosco V, Monterubbianesi R, Notarstefano V, Özcan M, Putignano A, Orsini G. 2021. ATR-FTIR, EDS and SEM evaluations of enamel structure after treatment with hydrogen peroxide bleaching agents loaded with nano-hydroxyapatite particles. **PeerJ 9:e10606 DOI 10.7717/peerj.10606**



Conclusion: The obtained results suggest that the application of the tested commercial bleaching agents, with a concentration of HP up to 12%, does not alter the morphological and chemical composition of the enamel surface and maintains its crystallinity.

Subjects Biochemistry, Biotechnology, Anatomy and Physiology, Dentistry, Drugs and Devices
Keywords SEM, ATR-FTIR spectroscopy, Hydrogen peroxide, Bleaching, Carbamide peroxide, Nano-hydroxyapatite

INTRODUCTION

In the last decades, patients' demand for aesthetic dental procedures, including bleaching, has considerably risen and, hence, tooth whitening has become an important topic in dentistry (*Demarco, Meireles & Masotti, 2009*).

Tooth color depends on both intrinsic and extrinsic stains (*Dabanoglu et al., 2009*). Intrinsic discolorations are caused by the incorporation of chromophores into dentin and enamel during odontogenesis or after an eruption, for example, hereditary disorders, tetracycline medication, excessive fluoride uptake or high fever associated with early childhood diseases (*Dabanoglu et al., 2009*; *Sherwood, 2010*). Extrinsic discolorations are the result of the adhesion on the tooth surface of chromophores, contained, for example, in coffee, tea, red wine, carrots, oranges, or tobacco. Since 1864, to avoid this inconvenience, whitening formulations, both at home and in-office, have been developed in dental practice. While most external stains can be removed by a thorough tooth cleaning, for correcting or at least reducing intrinsic discoloration and discoloration resistant to cleaning, the application of oxidizing agents, which directly break the double bonds of chromophores, is strongly required (*Watts & Addy, 2001*; *Dahl & Pallesen, 2003*; *Jin et al., 2013*). This procedure, referred to as bleaching, produces smaller molecules that absorb light with a shorter wavelength than visible light (such as ultraviolet light), and hence the teeth no longer appear to be colored (*Dadoun & Bartlett, 2003*; *Dabanoglu et al., 2009*).

Despite its popularity, there are severe side effects associated with the use of chemical oxidants, such as gingival irritation, modifications in surface morphology and tooth hypersensitivity (*Pintado-Palomino et al., 2015*; *Rodríguez-Martínez, Valiente & Sánchez-Martín, 2019*). The enamel structure plays an important role in the wellbeing of dental hard tissues; therefore, it is important to preserve it, even after treatments with bleaching agents (*Orilisi et al., 2020*). It is accepted that, under supervised clinical conditions, dental bleaching is safe for enamel integrity, but less is known about abusive and/or over-the-counter use (*Joiner et al., 2008*; *Demarco, Meireles & Masotti, 2009*; *Joiner, 2010*; *Silveira et al., 2018*). Some researchers have evaluated the effects of aggressive bleaching treatments on tooth structure, observing changes in the surface integrity and on the microstructure of enamel crystals, and a higher susceptibility to demineralization (*Cavalli et al., 2004*; *Shi et al., 2012*). Concerning the in-office products, they are commonly comprised of different concentrations of peroxide, varying from 5 to 40% of hydrogen



peroxide (HP) or carbamide peroxide (CP) (*Grazioli et al., 2018*). It is believed that HP penetrates enamel and dentin and, due to its chemical instability, it is decomposed into different active oxygen species under specific temperature, pH and light conditions (*Kwon & Wertz, 2015*); these free radicals may oxidize the conjugated chain of the chromophores (*Kwon & Wertz, 2015*), and, furthermore, the oxidation could promote morphological changes of the enamel (*Kawamoto & Tsujimoto, 2004*). On the other hand, CP dissociates into HP (approximately a third of its former concentration) and urea, which further breaks down into water and ammonia (*Da Silva Marques et al., 2012*; *Da Costa et al., 2012*). It has been ascertained that the risk of adverse effects increases with the peroxide concentration (*Perdigão, Baratieri & Arcari, 2004*). According to this finding, it is still necessary to develop a more effective, efficient, and long-lasting treatment to achieve an optimum whitening effect without any damage.

One of the last frontiers to overcome the demineralization is represented by the clinical application of nano-hydroxyapatite (nHA), within different carriers. Hydroxyapatite (HA) is a calcium phosphate compound with the molecular formula Ca_{10} (PO_4) OH_2 and a calcium-to-phosphorus ratio of 1:67. There are several forms of calcium phosphate in nature, but the most stable is represented by HA (*Bordea et al., 2020*). It can be considered as a revolutionary material due to its remarkable remineralizing effects on enamel initial lesions, it performs a protective action against caries and dental erosion (*Huang, Gao & Yu, 2009*). The nHA presents a strong affinity with demineralized surfaces due to its ability to bind to pores created by acid attacks. Indeed, after adhering, nHA multiplies and organizes into microclusters to form a uniform apatite layer that may completely overlap interprismatic and prismatic enamel (*Swarup & Rao, 2012*). According to these findings, in order to prevent hypersensitivity after bleaching, which can occur in a 70% of bleached patients, whitening gel has been enriched with HA in its nano form (*Kutuk et al., 2018*).

Therefore, this in vitro study investigates the effects on the enamel surface of the following three new commercial agents containing nHA and increasing amounts of HP: one at-home gel based on 6% HP, and two in-office ones, available at different concentrations of HP (12% and 18%). The effects of their application on human tooth surfaces were compared with the ones displayed by a commercially available CP-based, at-home bleaching gel (CONV). A non-treated group was also analyzed as control (CTRL). Surface enamel morphologies of all groups were qualitatively evaluated by Scanning Electron Microscopy (SEM), whereas Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Energy Dispersive X-ray Spectroscopy (EDS) were used to investigate respectively the effects of these bleaching agents on enamel chemical structure and elemental composition of the tooth surface, focusing on changes in the composition of Ca and P. The coupling of these analytical techniques represents a valuable tool to obtain a comprehensive overview of the morphological and chemical alterations of tooth surfaces. FTIR spectroscopy is a well-assessed method for studying the structural features in biomaterials (*Paschalis, Mendelsohn & Boskey, 2011*; *Kowalczuk & Pitucha, 2019*). By the analysis of the spectral profile and specific band area ratios, it is possible to evaluate the chemical composition of enamel and dentine and relate it to external



treatments and pathologies (*Verdelis et al., 2007*; *Lubarsky et al., 2012*; *Liu et al., 2014*). EDS determines the mineral content of dental hard tissues. The main advantage of this system is its capability to provide an accurate and non-destructive analysis of the specimens (*Furlan et al., 2017*; *Llena, Esteve & Forner, 2018*; *Kutuk et al., 2018*). As a consequence, this method was used to evaluate the changes in the mineral content of enamel. SEM analysis allows the visualization of images at high magnification and, hence, it is a useful approach for qualitative analysis of the enamel morphology (*Wang et al., 2013*).

The following two null hypotheses were tested: (1) the three bleaching agents based on HP and nHA do not affect the morphology and chemical composition of the enamel surface and display features similar to the CP-based agent (CONV); (2) the three bleaching agents based on HP and nHA do not affect the enamel structure.

MATERIALS AND METHODS

Teeth collection and samples preparation

Ten sound extracted third molars were collected, from subjects aged between 18 and 30 years (*Sulieman et al., 2004*; *Coceska et al., 2016*). Teeth were surgically extracted for orthodontic reasons at the Section of Stomatology of DiSCO Department, Polytechnic University of Marche, Ancona, Italy. According to the Local Ethics Committee guidelines and the 1964 Helsinki Declaration, informed consent was obtained from the subjects that were aware that their hard dental tissues, as discard of the surgical procedures, would be used for research purposes. After the surgical extraction, teeth were washed in an ultrasonic bath with distilled water for 2 min, in order to remove blood and biological remains, and then carefully examined to exclude the presence of lesions and decays, including hypoplastic defects and cracks: tooth exhibiting any of these features were excluded. Selected teeth were stored in a 0.5% (w/w) chloramine solution (NH_2Cl); immediately before the beginning of the application of the whitening products, they were cut, using a diamond saw (Buehler Isomet 1000, USA) with copious water irrigation, in mesiodistal direction to separate the buccal and lingual surfaces, starting at the level of the occlusal fissure.

Bleaching treatments

The following bleaching agents were in vitro tested: G1, at-home gel based on 6% HP (part A) and nano-hydroxyapatite (nHA, part B) in a ratio 1:3 (BioWhiten, Biodent Ltd., Istanbul, Turkey); G2, in-office gel based on 12% HP (part A) and nHA (part B) in a ratio 1:1 (BioWhiten, Biodent Ltd., Istanbul, Turkey); G3, in-office gel based on 18% HP (part A) and nHA (part B) in a ratio 3:1 (BioWhiten, Biodent Ltd., Istanbul, Turkey); CONV, commercially available at-home gel based on 10% CP (White Dental Beauty, Novon, Optident Optident, Ilkley, West Yorkshire). Part A and Part B of G1, G2 and G3 were mixed before application to obtain the above indicated HP and nHA ratios. Details about the bleaching agents (in terms of manufacturer's instructions and chemical composition) as well as the precise exposure times are given in Table 1. Teeth portions were randomly divided into five groups (n = 3 for each group) and submitted to the following treatments: CTRL (Control Group; untreated teeth); G1 (teeth treated with the



Table 1 Teeth experimental groups and chemical composition of bleaching agents.

Experimental groups	Manufacturer	Chemical composition	Exposure time
CTRL	None	None	None
G1	BioWhiten, Biodent Ltd., Istanbul, Turkey	Water, Glycerin, Alcohol, Sodium bicarbonate, Sodium hydroxide, 6% HP and nHA* (ratio 1:3)	50 min/day for 7 days
G2	BioWhiten, Biodent Ltd., Istanbul, Turkey	Water, Glycerin, Alcohol, Sodium bicarbonate, Sodium hydroxide, 12% HP and nHA (ratio 1:1)	10 min for 5 times
G3	BioWhiten, Biodent Ltd., Istanbul, Turkey	Water, Glycerin, Alcohol, Sodium bicarbonate, Sodium hydroxide, 18% HP and nHA* (ratio 3:1)	10 min for 5 times
CONV	White Dental Beauty, Novon, Optident Ilkley, West Yorkshire	10% CP and 0.25% w/w NaF (10% CP = ~3.3% HP)	2 h/day for 7 days

Note:
* nHA morphology; rod-like, width 5–20 nm (typically close to 10 nm) and length < 50 nm (typically between 20 and 40 nm).

G1 bleaching gel for 50 min/day for 7 days); G2 (teeth treated with the G2 bleaching gel for 10 min for five times); G3 (teeth treated with the G3 bleaching gel for 10 min for five times); CONV (teeth treated with CONV bleaching gel for 2 h/day for 7 days). Each bleaching treatment was performed according to the manufacturer's instructions. After each treatment, the bleaching agents were removed using a saliva aspirator and thoroughly rinsing the sample with distilled water. Between one application and another, samples were stored in distilled water at room temperature. In Fig. 1A a sketch of the experimental design is reported. All tooth samples were analysed by ATR-FTIR, SEM and EDS.

ATR-FTIR spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy measurements were carried out by using a Perkin Elmer Spectrum GX1 spectrophotometer, equipped with a diamond/ZnSe Attenuated Total Reflection (ATR) accessory. Teeth were accurately positioned on the crystal surface in order to obtain a good contact between the crystal itself and the external side of each tooth (not on the side exposed to cutting). On each sample, ten IR spectra were collected in the spectral range 4,000–650 cm^{-1}, with a spectral resolution of 4 cm^{-1}; each spectrum was the average of 64 scans. Raw IR spectra were converted in absorbance mode, 13-points smoothed, two-points baseline linear fitted, and then vector normalized in the entire spectral range (OPUS 7.1, Bruker Optics, Ettlingen, Germany). For each tooth portion, the average IR spectrum was calculated, together with the average ± standard deviation spectra, which were used to evaluate the homogeneity of sample spectra (OPUS 7.1). Average IR spectra were interpolated in the 1,800–650 cm^{-1} range, two-points baseline linear fitted, and then curve fitted by using the GRAMS/AI 9.1 software (Galactic Industries, Inc., Salem, NH, USA); a Gaussian algorithm was adopted. The center of the subcomponent bands was imposed based on second derivative minima positions (wavenumbers, cm^{-1}). To evaluate the goodness of the fitting, the spectral residual (obtained as the difference between the original trace and the fitted trace) was checked: fitting was considered satisfactory when this parameter was near to zero. For each subcomponent band, the position (in terms of wavenumbers, cm^{-1}), the integrated Area (A), and the Full Width at Half Maximum (FWHM) were

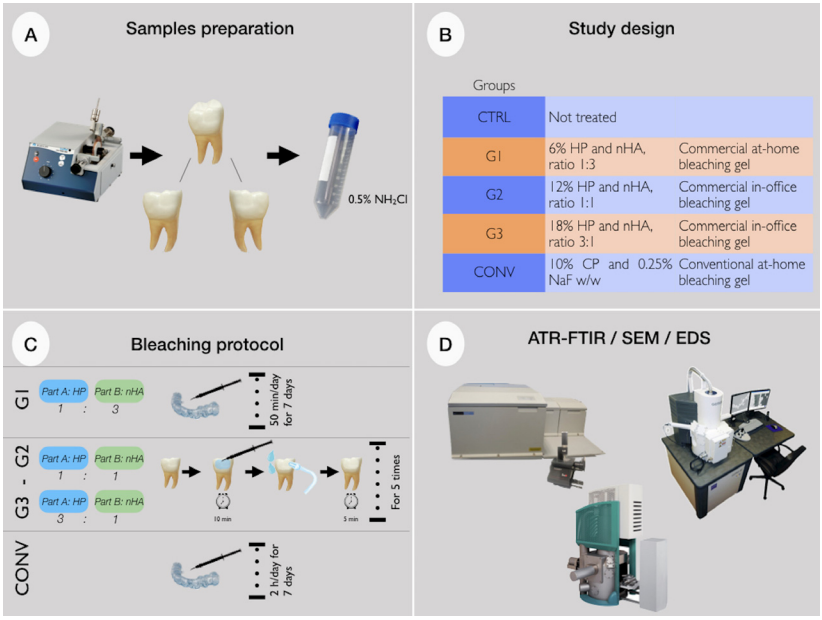

Figure 1 (A) Teeth samples preparation; (B) study design; (C) bleaching protocols; (D) ATR-FTIR spectroscopy, SEM microscopy and EDS evaluations. HP, hydrogen peroxide; CP, carbamide peroxide; nHA, nano-hydroxyapatite; ATR-FTIR, attenuated total reflectance-fourier transform infrared spectroscopy; SEM, scanning electron microscope; EDS, energy dispersive X-ray spectroscopy.
Full-size DOI: 10.7717/peerj.10606/fig-1

determined. The assignment of IR peaks was performed according to literature (*Gadaleta et al., 1996*; *Boskey & Mendelsohn, 2005*). Specific band area ratios were calculated.

Scanning Electron Microscopy

Scanning Electron Microscopy observations were carried out by a Zeiss Supra 40 field-emission electron microscope (Polytechnic University of Marche, Department of Materials, Environmental Science and Urban Planning (SIMAU)). The same samples analysed by ATR-FTIR, a non-destroying analytical technique, were assembled in a sample holder and metallized with vacuum precipitation of a gold film on the dental surface. Scanning electron micrographs of the enamel were obtained with magnifications of 400× and 2,000×. SEM operated at 30 kV and at a 12 mm working distance. The obtained micrographs were evaluated descriptively, observing the variations in the micromorphology of the dental enamel of all different analysed groups.

Energy Dispersive X-ray Spectroscopy

The enamel chemical characterization of the samples was carried out by EDS, performed by the Scanning Electron Microscope equipped with microanalysis EDAX Element

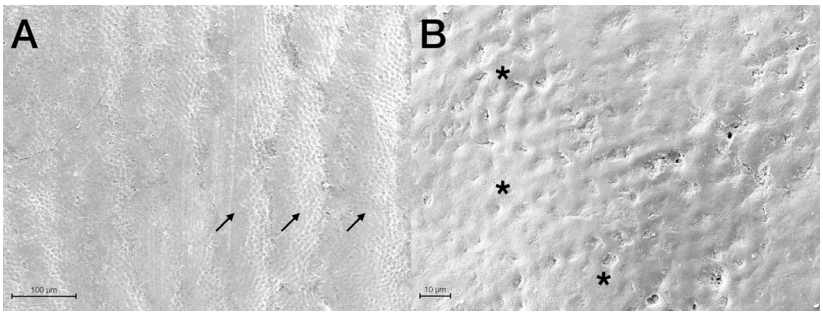

Figure 2 Scanning electron micrographs of a representative CTRL tooth sample at (A) 400× and (B) 2,000× original magnification. The micrograph A presents the typical aspect of sound enamel morphology, with enamel-like crystal structure and perikymata (black arrows). The micrographs B displays a homogeneous smooth area between the interprismatic rods (asterisks).

Full-size DOI: 10.7717/peerj.10606/fig-2

(TESCAN VEGA3). The same samples were used for SEM and EDS. The operating parameters were: 15 mm working distance, 25 kV accelerating voltage, and magnification of 400×. The concentrations by weight (%) of the following chemical elements were evaluated: Oxygen (O), Fluorine (F), Sodium (Na), Phosphorous (P), Chlorine (Cl) and Calcium (Ca).

Statistical analysis

Data deriving from ATR-FTIR and EDS analyses were presented as mean ± standard deviation (SD). Significant differences between experimental groups were determined by means of Kruskal–Wallis test, followed by Dunn's test, using the statistical software package Prism6 (GraphPad Software, Inc., San Diego, CA, USA). The group size was set at $N = 3$ for all the experimental groups; significance was set at $p < 0.05$. Adjusted p values were calculated for each multiple comparison and reported in Tables S1 and S2.

RESULTS

Scanning Electron Microscopy analysis was exploited to investigate and compare the surface morphology of each treated group with that of untreated one (CTRL). Figures 2–6 showed the scanning electron micrographs of the enamel surface of representative tooth samples subjected to the different bleaching treatments proposed. Although at low magnification (400×), all micrographs of each bleached group showed apparently intact surfaces, similar to those of CTRL, at high magnification (2,000×) differences among groups were observed. In Figs. 2A and 2B, the micrographs of a representative CTRL tooth sample are reported; although the surface is not completely smoothened, the presence of aprismatic enamel and perikymata can be observed, suggesting that the physiological structure is preserved. Conversely, in all teeth samples submitted to bleaching (Figs. 3–6), signs of the treatments were randomly observed on the enamel surface, even though different types of defects and distinct severity of such events were found. Indeed, all G1 samples displayed a sound and intact enamel surface similar to that

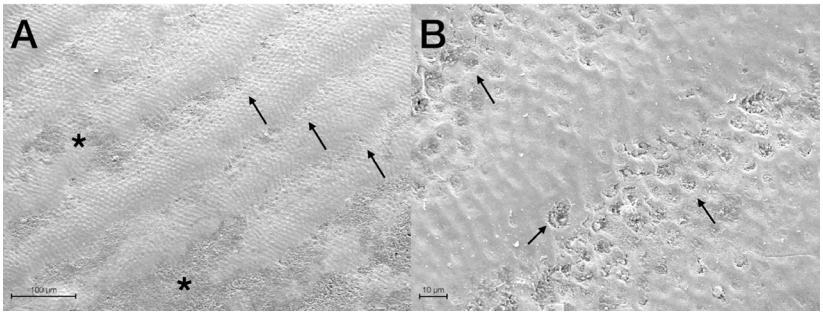

Figure 3 Scanning electron micrographs of a representative G1 tooth sample at (A) 400× and (B) 2,000× original magnification. The micrograph A shows a sound enamel morphology with enamel-like crystal structure and perikymata (black arrows), within minimal loss of enamel surface integrity (black asterisks). The micrograph B highlights well-preserved interprismatic rods and a slight dissolution of prism cores (black arrows). Full-size DOI: 10.7717/peerj.10606/fig-3

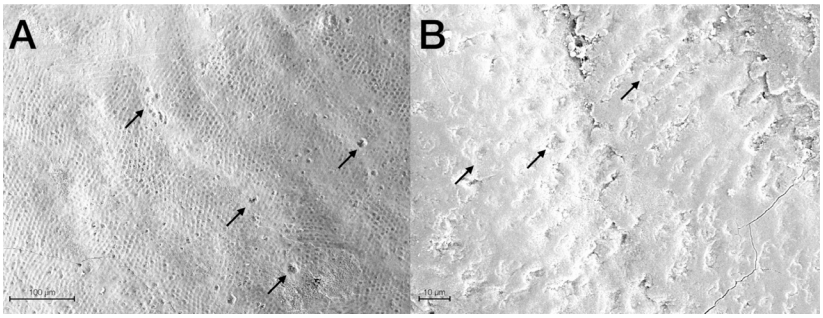

Figure 4 Scanning electron micrographs of a representative G2 tooth sample at (A) 400× and (B) 2,000× original magnification. The micrograph A presents a minimal loss of surface integrity with the presence of micropores (black arrows), partial loss of perikymata and raised enamel interrods. The micrograph B highlights the partial core prisms dissolution (black arrows) by the raised interrod structure. Full-size DOI: 10.7717/peerj.10606/fig-4

of CTRL, except one, which revealed a minimal loss of integrity, with an increase of enamel irregularities (Figs. 3A and 3B). Also, in G2, most scanning electron micrographs showed slight changes on the surface, with the presence of microporosities, but only in one sample, at higher magnification, an irregular surface pattern, with partial dissolution of the prisms, was observed (Figs. 4A and 4B). With the increase in the percentage of HP, morphological surface alterations became much more pronounced. In fact, changes in the enamel bleached surface were well evident in G3; in this group, the same pattern of alterations already observed in G2 was found, but in a more severe manner, together with an increased number of microporosities on the enamel surface (Figs. 5A and 5B). Moreover, in G3, the dissolution of prism cores was often present, together with partial micro-erosions in the surface (Fig. 5B). In CONV minimal defects, similar to those found in G1 samples, were detected. In particular, the micrographs showed a slight

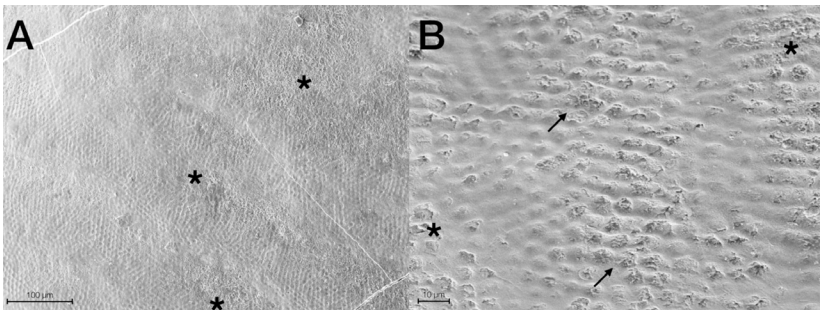

Figure 5 Scanning electron micrographs of a representative G3 tooth sample at (A) 400× and (B) 2,000× original magnification. The micrograph A displays a partial loss of surface integrity with some enamel micro-erosion (black asterisks), loss of perikymata and raised interrods. The micrograph B shows different enamel micro-erosions (black asterisks), associated with partial prism core dissolution and slight disorganization of interprismatic rods structure (black arrows).
Full-size DOI: 10.7717/peerj.10606/fig-5

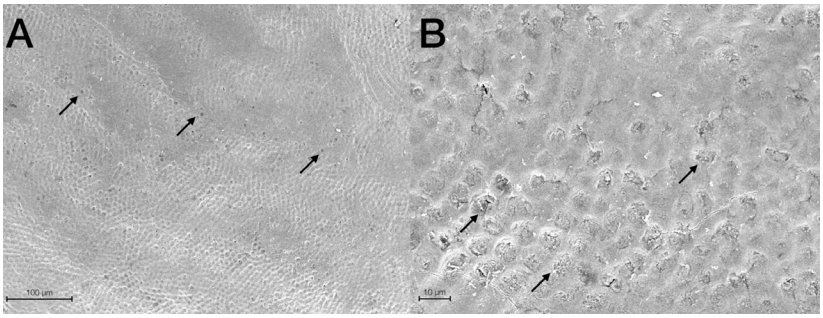

Figure 6 Scanning electron micrographs of a representative CONV tooth sample at (A) 400× and (B) 2,000× original magnification. The micrograph A shows a sound enamel morphology, with enamel-like crystal structures and minimal loss of surface integrity; some micropores (black arrows), loss of perikymata and raised enamel interrods are also identified. The micrograph B highlights well-preserved interprismatic rods, although minimal enamel erosion and slight prism cores dissolution (black arrows) are present.
Full-size DOI: 10.7717/peerj.10606/fig-6

increase of irregularities, with a partial loss of the enamel surface integrity, and a little occurrence of microporosities (Figs. 6A and 6B).

ATR-FTIR spectroscopy was exploited to analyze the changes in the chemical structure and composition of tooth mineralized tissues. In Fig. 7, the average IR spectra of all experimental groups are reported; three areas assigned to the organic matrix (Zone 1), to carbonate groups and, to a lesser extent, to protein side chains (Zone 2), and to phosphates (Zone 3) are showed. A similar spectral profile was found in CTRL, G1, G2 and CONV spectra, while a different shape was showed by G3 samples. Due to the presence of several convoluted bands, average IR spectra of CTRL, G1, G2, G3 and CONV groups were curve fitted in the 1,800–650 cm^{-1} spectral range based on the position of the minima of

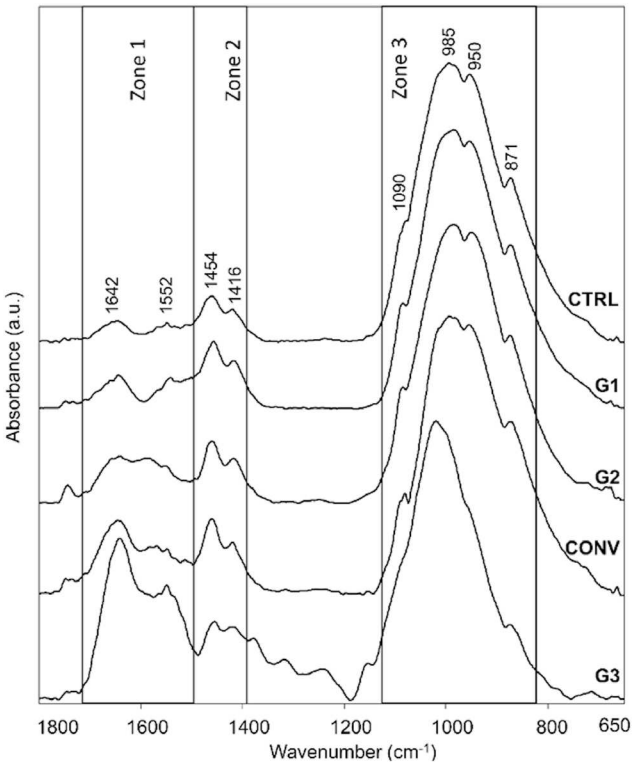

Figure 7 Average IR spectra of CTRL, G1, G2, CONV and G3 teeth samples. Spectra are showed in the 1,800–650 cm^{-1} spectral range and are shifted along *y*-axis for a better understanding of the figure. The position of the most meaningful IR bands of CTRL samples is reported in terms of wavenumbers (cm^{-1}). Black boxes indicate the spectral ranges related to the organic (Zone 1) and inorganic (carbonates, Zone 2 and phosphates, Zone 3) matrices. Full-size DOI: 10.7717/peerj.10606/fig-7

second derivative spectra, to detect all the subcomponent bands. CTRL, G1, G2 and, as further extent, CONV spectra were characterized by prominent bands related to the phosphates groups of hydroxyapatite (HA) (~1,090 cm^{-1}, ~1,026 cm^{-1}, ~984 cm^{-1} and ~939 cm^{-1}), and to those attributable to carbonates (~1,462 cm^{-1}, ~1,416 cm^{-1} and ~869 cm^{-1}); weak peaks related to the organic matrix (~1,649 cm^{-1} and ~1,552 cm^{-1}, Amide I and II bands of proteins) were also detected. Spectra of G3 samples were characterized by the same peaks, with an increase of the intensity of the bands attributable to the organic matrix. The statistical analysis of meaningful band area ratios was performed by Kruskal–Wallis test, followed by Dunn's test (group size $N = 3$ for each experimental group; significance $p < 0.05$) (Fig. 8). According to the ratio $A_{984}+A_{1090}/A_{1649}$ (ν1+ν3 PO_4/Amide I), similar values (not statistically significant, $p > 0.05$) were found in CTRL, G1 and G2 groups; G3 showed a statistically significant decrease of this ratio ($p < 0.05$), while an intermediate value was observed in CONV ($p > 0.05$). A similar trend

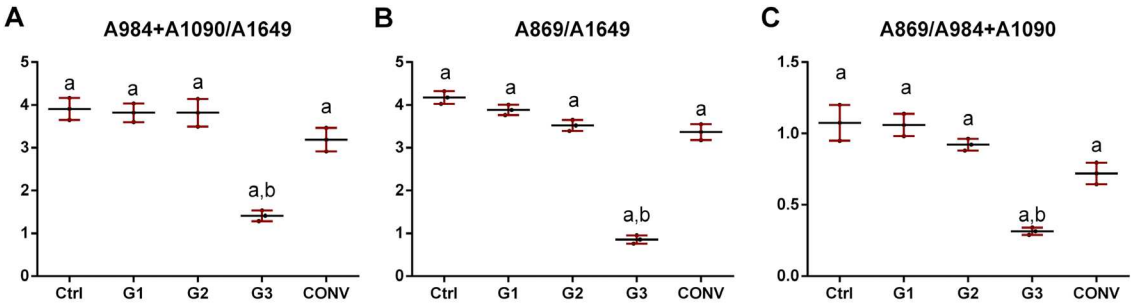

Figure 8 Statistical analysis of the following band area ratios. (A) A984+A1090/A1649 (ν1+ν3 PO_4/Amide I); (B) A869/A1649 (nCO_3/Amide I) and (C) A869/A984+A1090 (nCO_3/ν1+ν3 PO_4). Data are reported as mean ± S.D. Different letters indicate statistically significant differences among groups ($p < 0.05$).
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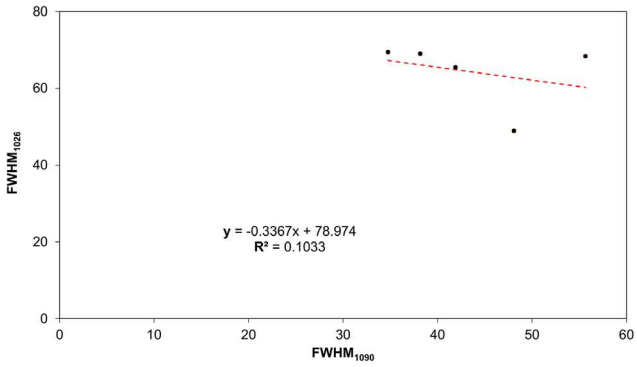

Figure 9 Correlation between $FWHM_{1090}$ and $FWHM_{1026}$, using the coefficient of determination (R^2). Points indicate respectively from left to right: CTRL, G1, G2, G3 and CONV.
Full-size DOI: 10.7717/peerj.10606/fig-9

was observed in the band area ratio A_{869}/A_{1649} (ν CO_3/Amide I) (carbonates/matrix ratio), with no differences between CTRL and G1 samples ($p > 0.05$); conversely, the lowest value was found in G3 ($p < 0.05$); finally, G2 and CONV showed a small reduction of this ratio ($p > 0.05$). As regards the band area ratio $A_{869}/A_{984}+A_{1090}$ (ν CO_3/ν1+ν3 PO_4), similar or slightly lower values were observed in CTRL, G1 and G2 groups ($p > 0.05$); also, in this case, G3 showed the lowest value ($p < 0.05$), while CONV exhibited an intermediate behavior ($p > 0.05$). In Fig. 9, the correlation between the Full Width at Half Maximum (FWHM) values of these two bands is reported. A linear correlation was found among all the experimental groups ($R^2 = 0.1033$), with R^2 improving if excluding G3 ($y = -0.56x + 89.50$; $R^2 = 0.8457$). However, the presence of a negative slope, both including and excluding G3, indicates that bleaching treatments cause an increase of the amorphous hydroxyapatite.

For all the groups, the chemical composition of dental enamel in terms of relative amounts of O, F, Na, P, Cl and Ca elements, was analysed using EDS (Table 2). The results



Table 2 The chemical composition of dental enamel was analyzed in all groups using EDS. The results showed low alterations in the levels of chemical constituents. Variations of the concentration values by weight (%) of the chemical elements observed by means (S.D.) of Spectroscopy of Dispersion of Ray-x (EDS) of dental enamel submitted to different bleaching agents.

	CTRL	G1	G2	G3	CONV
O	30.41 (8.18)	42.48 (4.77)	35.27 (15.64)	38.85 (5.38)	42.30 (3.27)
F	0.59 (0.36)	0.94 (1.13)	0.52 (0.49)	0.31 (0.16)	0.37 (0.10)
Na	0.60 (0.28)	0.94 (0.08)	19.03 (0.60)	19.19 (0.19)	1.00 (0.10)
P	20.81 (0.91)	18.34 (1.65)	19.03 (1.68)	19.19 (1.14)	18.23 (0.70)
Cl	0.56 (0.03)	0.50 (0.08)	0.55 (0.13)	0.52 (0.04)	0.61 (0.02)
Ca	47.03 (7.92)	36.80 (4.06)	43.78 (14.91)	40.33 (4.31)	37.49 (2.71)

showed only low alterations in the levels of the following chemical constituents: (i) slightly higher levels of O in bleached samples (G1, G2, G3 and CONV) with respect to CTRL (Fig. 10A); (ii) lower levels of F in G2 and G3 samples (Fig. 10B); (iii) no statistically significant difference in Na, P, and, as further extent, Cl amounts in all groups (Figs. 10C–10E) and (iv) lower levels of Ca in G1, G2 and G3 groups (Fig. 10F). A constant ratio between Ca and P was found in all experimental groups, with a slight but not significant decrease in G2 (Fig. 11).

DISCUSSION

In the last decades, the use of bleaching agents has been greatly increased. Due to this demand, a large variety of products has been developed (*Pinto et al., 2014*). One of the possible side-effects of bleaching treatments is that enamel structure may be weakened by the oxidation of the organic or inorganic elements. Several studies have investigated the effects of these bleaching agents in terms of mineral loss, degree of demineralization and modifications in the morphology of tooth surface, but to date, the results are quite contradictory (*Shannon et al., 1993*; *Bitter & Sanders, 1993*; *Ernst, Marroquín & Willershausen-Zönnchen, 1996*; *Oltu & Gürgan, 2000*). A study, published in 2000, examined the effects on the enamel structure of three bleaching agents containing 10%, 16% and 35% of CP (*Oltu & Gürgan, 2000*). The results revealed that 10% or 16% of CP did not seem to affect the structure of enamel, whereas the highest concentration caused severe damages. These results were also confirmed by a recent study, in which authors found that bleaching gel based on 10% of CP was substantially safe, but, when used for a time that exceeds that recommended by the manufacturer, could be able to reduce the micro-hardness, modify the ultrastructure and promote changes in the enamel chemical composition, only (*Vilhena et al., 2019*). Furthermore, Fearon et al. compared a number of studies of different whitening procedures and concluded that changes in the tooth surface structure and increased tooth sensitivity can occur, especially if highly concentrated HP and CP solutions are applied (*Fearon, 2007*). Moreover, some authors reported that 15% of HP is the maximum effective concentration to avoid enamel morphological alterations; higher concentrations do not improve the whitening effect, but rather increase the possibility of enamel damages. In fact, changes in the surface

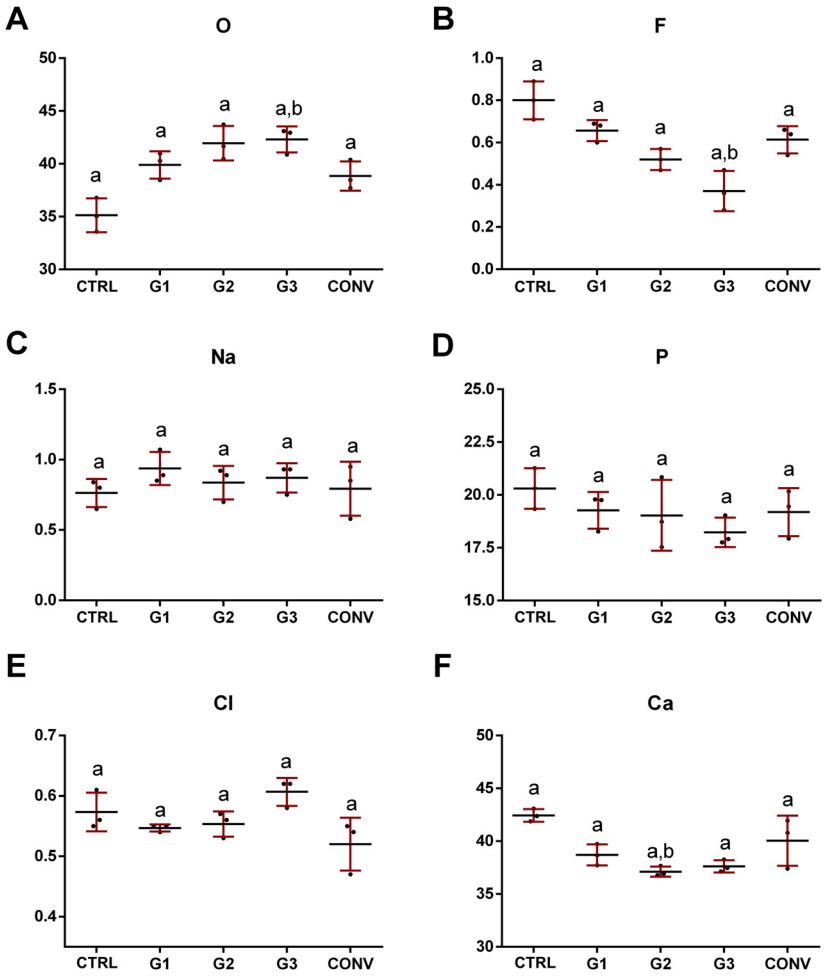

Figure 10 Mean values and standard deviation by weight (%) of different elements contained in the analyzed samples. (A) Oxygen, O; (B) Fluorine, F; (C) Sodium, Na; (D) Phosphorous, P; (E) Chlorine, Cl; (F) Calcium, Ca. Different lowercase letters indicate statistically significant differences among groups ($p < 0.05$). Full-size DOI: 10.7717/peerj.10606/fig-10

morphology reduces hardness properties and increases roughness (*Loguercio et al., 2017*; *Grazioli et al., 2018*; *Rodríguez-Martínez, Valiente & Sánchez-Martín, 2019*). Hence, in addition to the conventional bleaching materials based on various peroxide compounds, which may have some detrimental effects, scientists have been searching for alternative materials (*Dabanoglu et al., 2009*; *Llena et al., 2019*). Conversely, there is limited information discussing the use of low concentrated bleaching agents as in-office treatments and more scientific evidence should be sought on this topic. Caution is required when



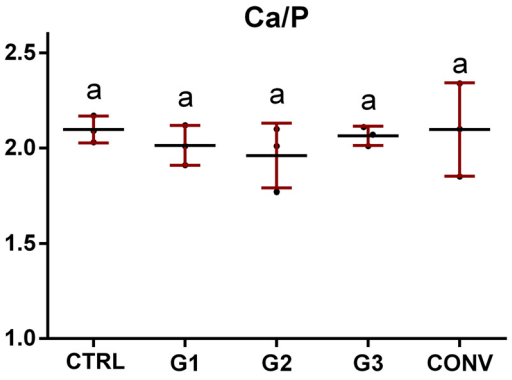

Figure 11 Mean values and standard deviation of Ca/P ratio, according to the treatment groups. Mean values of Ca/P ratio. Different letters indicate statistically significant differences among groups ($p < 0.05$). Full-size DOI: 10.7717/peerj.10606/fig-11

performing in-office bleaching, following the exact manufacturer instruction, according to the percentage and the time of application of the selected bleaching gel. Indeed, according to recent clinical studies, the current strategies to avoid the discomfort and damages associated with bleaching treatments are not totally effective, and they are not being optimized yet (*Vaez et al., 2018*; *Rodríguez-Martínez, Valiente & Sánchez-Martín, 2019*; *Epple, Meyer & Enax, 2019*).

This is the first in vitro study reporting ATR-FTIR, SEM, and EDS evaluation of the effects and safety of newly commercialized bleaching agents containing nHA on human third molars. The association of these techniques allows to achieve a robust correlation between imaging, spectral and chemical data, providing reliable and objective information both on the morphology and the chemical composition of the analysed samples. To improve the understanding of dental enamel structure, this approach was particularly useful, giving the opportunity to evaluate the effect of bleaching agents from different point of view.

Although the teeth were in dysodontiasis, electron micrographs of CTRL showed sound mineralized enamel surfaces without altered features, resembling the ones of normally mature post-erupted teeth (*Palti et al., 2008*; *Markovic et al., 2010*). The effects on the enamel surface of the three tested agents newly-launched on the market, with low and medium percentages of HP, loaded with nHA at different ratios were evaluated and compared with those of a 10% CP- based bleaching agent, selected for its well-documented safety and efficacy (*Meireles et al., 2008*; *Türkün et al., 2010*; *Basting et al., 2012*; *Eachempati et al., 2018*). Enamel morphological features and chemical composition of the treated groups were compared with those of a non-treated group. Findings described in this study agree with those already reported in the scientific literature. Indeed, several studies detected significant changes on the enamel surface after bleaching with concentrated 35% HP or CP, whereas 10% and 16% concentration did not alter the enamel



surface (*Oltu & Gürgan, 2000*; *Sulieman, Addy & Rees, 2003*). In particular, recent studies reported that bleaching gel containing 15% of HP showed greater/neutral pH, better pH stability, lower hardness loss, and no surface damage to enamel than the 25–35% HP-based gels (*Abouassi, Wolkewitz & Hahn, 2011*; *Magalhães et al., 2012*; *Grazioli et al., 2018*).

Scanning Electron Microscopy analysis demonstrates that the at-home and the low percentage of the in-office bleaching agents do not seem to affect the enamel surface morphology, especially when observed at low magnification (400×). Scanning electron micrographs of CTRL teeth display the sound physiological pattern of the enamel surface, marked by the presence of the aprismatic enamel. Similarly, no significant changes in enamel structure are depicted in G1, exposed to a relatively low concentration of HP. The pattern observed in G2 is analogous to CONV, with generally common preserved features as in CTRL and G1, except in one sample which has revealed a slight increase of the enamel irregularities, with a partial loss of the interprismatic spaces and perikymata, and some microporosities. On the other hand, SEM images of G3 display a marked depth of enamel irregularities, removal of the aprismatic layer, and an evident presence of microporosities. Similar altered features have been demonstrated by other studies, in which authors concluded that gels with 25% and 35% of HP produced morphological changes on enamel, with prism rods exposure; conversely, the gel containing 15% of HP produced a similar topography to that obtained for the non-bleached specimen (*Miranda et al., 2005*; *Kwon & Wertz, 2015*; *Vilhena et al., 2019*). These findings are consistent with the study by *Bistey et al. (2007)* stating that bleaching agents based on 10–30% HP, without nHA, can provoke severe morphological alterations on the enamel surface.

Scanning Electron Microscopy results have been largely confirmed by ATR-FTIR spectroscopy. Indeed, comparable spectral profiles were found in the average IR spectra of CTRL, G1, G2 and CONV, focused on the ratio $A_{984}+A_{1090}/A_{1649}$ (ν1+ν3 PO_4/Amide I), indicated as mineral/matrix ratio, expressing the degree of mineralization of dental tissues (*Boskey & Mendelsohn, 2005*; *Boskey et al., 2005*). Conversely, G3 spectra reveal an increase of the amorphous HA and, as a further extent, of the organic matrix (*Lubarsky et al., 2012*). The peaks at 1,090 cm^{-1} and 1,026 cm^{-1} are usually referred to as phosphate groups in poor and well-crystallized HA (*Farlay et al., 2010*). The different shapes of the IR bands of phosphates of HA (*Gadaleta et al., 1996*), confirm the lack of mineral in enamel, as visualized by SEM microporosities.

Energy Dispersive X-ray spectroscopy is a precise and non-destructive technique for studying the mineral component of enamel (*Llena, Esteve & Forner, 2018*). It involves sample bombardment with a high-voltage electron beam that generates different wavelengths for each type of mineral. The changes in the wavelength of the radiation emitted by the sample indicate changes in its mineral concentration (*Llena, Esteve & Forner, 2018*). The obtained results revealed slight but not statistically significant differences ($p > 0.05$) in the concentration values by weight (%) of O, F and Ca elements among the experimental groups, while similar amounts were observed as regards Phosphorous and Sodium ($p > 0.05$). In particular, a slight increase in O concentration was



found after the application of the tested bleaching agents. This fact is probably associated to the action of the peroxide on the mineralized enamel structure, a reaction well mentioned by the scientific literature (*Cavalli, De Carvalho & Giannini, 2005*; *Godinho et al., 2014*; *Silveira et al., 2015*; *Vilhena et al., 2019*). As regards P, a trend similar to that observed for Ca, even if not statistically significant ($p > 0.05$), was pinpointed. In fact, similar amounts of Ca were observed in CTRL and CONV ($p > 0.05$), while a slight decreasing trend was found in G1, G2 and G3 groups ($p > 0.05$), reflecting the increasing percentage of HP in the bleaching treatments. The relationship between Ca and P is an essential indicator of the remineralization process (*Cakir et al., 2011*; *Castro et al., 2016*). The Ca/P ratio calculated in this study did not show any statistically significant change in all the experimental groups ($p > 0.05$) (*Vilhena et al., 2019*). It is known that high concentrations of HP can modify the Ca/P ratio (*Cavalli et al., 2010*) and the decreases in the level of the two elements could lead to an irreversible alteration, preventing the remineralization process from occurring. All these findings suggested that the percentages of bleaching agents adopted in this study were not sufficient to cause relevant chemical alterations of the enamel (*Potocnik, Kosec & Gaspersic, 2000*). Therefore, according to these results, the first null hypothesis is partially rejected.
As regards F values, a decreasing trend was observed when high percentages of HP are used. Similar values close to CTRL were found in G1 and CONV. G1 levels can be explained by the low percentage of HP, while those of CONV by the use of an F-containing bleaching agent. All these results suggest that low concentrated gels may be used more effectively and safely than high-concentrated agents, also for in-office bleaching procedures. Indeed, G2 seems to not significatively alter the enamel surface both qualitatively and quantitatively. On the other hand, some alterations on the enamel surface could be highlighted, as previously described for G3 (*Llena, Esteve & Forner, 2017*).

Existing scientific literature has well demonstrated that nHA plays a crucial role in repairing the altered enamel morphology, thanks to its major advantages as the analogy to the mineral structure of teeth, bioactivity, and biocompatibility (*Vandiver et al., 2005*; *Pepla et al., 2014*; *Nozari et al., 2017*). The nHA shows a strong affinity with demineralized surfaces and it is able to bind to pores created by demineralization (*Li et al., 2008*; *Swarup & Rao, 2012*). Chen et al. described the mechanism of enamel prism formation in presence of nHA. During the demineralization, nHA particles can gather together to form a stable prismatic structure such as the one observed in the enamel, thus preserving its crystallinity (*Chen et al., 2005*). Moreover, the remineralizing and repairing potential of nHA was also evaluated with positive outcomes in previous studies (*Lelli et al., 2014*; *Vano et al., 2015*). However, in partial disagreement with the study by *Bistey et al. (2007)*, consistent to our results, it can be concluded that not only the 6% HP/nHA—based agent, but also the 12% HP/nHA gel did not show changes on enamel crystallinity. Indeed, SEM observations demonstrate that bleaching agents based on 6% and 12% HP, coupled with nHA, at 1:3 and 1:1, respectively, basically preserve the interprismatic enamel structure. Moreover, no changes are observed in the chemical composition of G1 and G2 when compared to CTRL. Conversely, G3 samples show an increase of the amorphous HA component of the enamel, probably ascribable to a lower amount of nHA



in the bleaching gel with respect to the others. Moreover, the nHA contained in the bleaching gel used for G3 does not seem to be combined with the HA of the enamel surface, probably due to its lower pH value. Indeed, as reported by the manufacturer, the pH values of G1, G2 and G3 bleaching gels are respectively 9.2, 7.8 and 6.4, G1 and G2 showing an alkaline pH, while G3 being close to neutral. Thus it can be suggested that pH values may be relevant, as also described by several studies (*Azrak et al., 2010*; *Basting et al., 2012*; *De Geus et al., 2016*; *Grazioli et al., 2018*). CONV, containing 10% CP, is approximately equal to 3.3% HP (CP/HP ratio is 1/3) (*Eachempati et al., 2018*). It would be expected that a higher concentration of HP could lead to lower HA crystallinity than CONV 3.3% HP. On the contrary, our findings show that G1 and G2 have similar HA crystallinity of CONV and CTRL. Thus, the second null hypothesis can be rejected.

The absence of using saliva as a remineralizing agent after bleaching could be a limitation of our study, since saliva plays an important role in protecting the substrate from excessive mineral loss, allowing enamel remineralization (*Do Amaral et al., 2012*; *Grazioli et al., 2018*). Indeed, saliva, containing phosphate, allows to revert the effects generated by the bleaching process and to play as an alkalizing agent (*Vargas-Koudriavtsev & Herrera-Sancho, 2017*). However, according to other reports, since this study aims to analyze the morphology and chemical composition of the enamel surface after using bleaching agents, the teeth between each bleaching agent application were washed and immersed in distilled water in order to not alter the enamel and to not introduce a new uncontrolled variable in the research (*Sulieman et al., 2004*; *Giulio et al., 2009*; *Jin et al., 2013*; *Vargas-Koudriavtsev & Herrera-Sancho, 2017*; *Farawati et al., 2019*; *Vilhena et al., 2019*), while chloramine solution was used for their collection and maintenance (*Grazioli et al., 2018*; *Silveira et al., 2018*). Moreover, since the Ca/P ratio observed in all groups was not different from CTRL ($p > 0.05$), it can be suggested that no significant demineralization has occurred. Another limitation could be the limited range of the bleaching agents concentration analyzed, that, however, was selected among the most commonly used ones (*Eachempati et al., 2018*). Although some authors have claimed that microhardness test can be a reliable method to better understand the changes of the enamel physical properties (*Pepla et al., 2014*; *Grazioli et al., 2018*), even the effect of chemical alterations - as evaluated by our spectroscopic analyses - can be considered as a reliable evaluation on the physical-mechanical properties of some bleaching agents (*González-López et al., 2016*; *Vargas-Koudriavtsev & Herrera-Sancho, 2017*). Finally, further investigations might evaluate the color changes after the use of bleaching agents containing nHA and based on different HP concentrations, so as to recommend bleaching treatments that could give excellent aesthetic results without tissue damage. In this context, also clinical trials evaluating post-bleaching tooth sensitivity could be highly recommended.

CONCLUSIONS

From this in vitro study, it can be concluded that the tested commercial bleaching agents, in particular the 10% CP and the 6% HP at-home, and the 12% HP in-office, could be safely used by dental clinicians, following the manufacturer protocol, since they neither



chemically alter nor microscopically change the enamel surface. In order to better evaluate this latter topic and to improve knowledge on the mechanism of (re)action of these commercial bleaching gels on dental enamel as well as on the oral environment, it would be interesting to further propose the evaluation of these whitening agents with and without saliva interaction.

ACKNOWLEDGEMENTS

The authors thank the manufacturer BiODENT MEDICAL (Kadiköy, Istanbul, TR) and Optident Ltd. (Valley Drive, Ilkley, UK) for providing the bleaching material support. The authors extend their gratitude to Adriano Di Cristoforo and Carla Conti for their precious support in SEM and ATR-FTIR measurements.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

The authors received no funding for this work.

Competing Interests

Giovanna Orsini is an Academic Editor for PeerJ.

Author Contributions

- Giulia Orilisi conceived and designed the experiments, performed the experiments, prepared figures and tables, and approved the final draft.
- Vincenzo Tosco conceived and designed the experiments, performed the experiments, prepared figures and tables, and approved the final draft.
- Riccardo Monterubbianesi performed the experiments, reviewed the drafts of the paper, and approved the final draft.
- Valentina Notarstefano performed the experiments, analyzed the data, prepared figures and tables, and approved the final draft.
- Mutlu Özcan conceived and designed the experiments, authored the drafts of the paper, and approved the final draft.
- Angelo Putignano conceived and designed the experiments, authored and reviewed the drafts of the paper, supervised the entire project and approved the final draft.
- Giovanna Orsini conceived and designed the experiments, analyzed the data, authored and reviewed the drafts of the paper and approved the final draft.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Ten sound extracted third molars were collected, from subjects aged between 18 and 30 years. Teeth were surgically extracted for orthodontic reasons at the Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy. According to the Local Ethic Committee guidelines and the 1964 Helsinki Declaration,



written informed consent was obtained from the subjects that were aware that their hard dental tissues, as discard of the surgical procedures, would be used for research purposes.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.10606#supplemental-information.

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3. Nanotechnological Application in Restorative Dentistry

Restorative dentistry has traditionally focused on the use of dental materials to replace the infected tooth structure in the oral environment in order to restore the physical function following the tissue loss from disease processes. It is estimated that more than 290 million restorations are placed each year worldwide (154), representing one of the most prevalent medical intervention in the human body (70). Resin-based composites (RBC) have become the gold standard in restorative dentistry for their fundamental role in guaranteeing excellent aesthetics and function. RBC are based on the combination of three main chemically different components: the organic matrix (a synthetic monomer or resin), the inorganic matrix (the filler), and a coupling agent (usually silane) to bond the filler to the organic matrix. Moreover, RBC present activators, as the camphoroquinone, that producing free radicals promote light-activated polymerization of the organic matrix.

Matrix: The most commonly organic matrix used in commercially available RBC is the Bis-GMA (bisphenolglycidil methacrylate) monomer. Other base monomers used in current RBC include triethyleneglycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA), ethoxylated bisphenol-A-dimethacrylate (Bis-EMA), decanediol dimethacrylate (D3MA), bis(methacryloyloxymethyl) tricyclodecane, and urethane tetramethacrylate (UTMA). Bis-GMA has a very high viscosity due to the hydrogen bonding interactions that occur between the hydroxyl groups and the monomer molecules. Therefore, Bis-GMA must be diluted with more fluid monomers to provide the proper viscosity for a correct and manageable utilization of RBC (52). TEGDMA, due to its viscosity and excellent copolymerization characteristics, is normally used as diluent monomer, in particular for UDMA and Bis-GMA-based composites, to provide a fluid resin, which can be maximally filled with inorganic particles (24).



Filler: RBC contain a wide range of fillers, including silica, quartz and radiopaque silicate particles, based on the oxides of barium, strontium, zinc, aluminium and zirconium. These fillers increase the strength and the modulus of elasticity, thus reducing polymerization shrinkage, the coefficient of thermal expansion, and water absorption.

Silane Coupling Agents: The formation of a strong covalent bond between inorganic fillers and the organic matrix is essential in order to obtain improved mechanical properties in RBC. Bonding of these two phases is achieved by coating the fillers with a silane coupling agent, that has functional groups to chemically link the filler and the matrix. A typical coupling agent is 3-methacryloxypropyltri-methoxysilane (MPTS). One end of the molecule can be bonded to the hydroxyl groups of silica particles, and the other end is capable of copolymerizing into the polymer matrix (25).

Each of these components can be modified depending on their clinical application, and the resultant combination determines the physical, chemical, mechanical and optical properties of materials. The introduction of novel materials in the last few years has led to realize RBC with improved properties, being nanoparticles-based fillers the main innovation in the arena of dental restorative materials.

The RBC classification has evolved over the years, generally based on filler-size distribution, filler content or composition. The widely accepted classification defines RBC in macro-filler composites (0.1-100 μm), micro-filler composites (0.04-0.1 μm) and hybrid composites (fillers of different sizes) (111). In addition, thanks to the nanotechnological application on RBCs, Randolph et al. provide a modern classification based on the introduction of nano-filler and nano-hybrid composites (142). This terminology refers to materials containing a fraction of



nanoparticles (<100 nm) and of sub-micrometric particles (≤1 μm, typically averaging 0.5–1.0 μm) (52). The chemistry, morphology and the size of filler particles used in RBC differ considerably, even within the nanoscale. The nanofillers, regardless of the type, are incorporated into the resin, in order to improve the properties optimising the performance of the final restorative material. Indeed, the fillers addition enhances the mechanical properties of composites, reduces the polymerisation shrinkage, modifies the thermal expansion coefficient of the composite to match that of the tooth, improves handling provides radio-opacity and provide the composites with wear resistance and translucency (71).The smaller particle size causes less curing shrinkage and viscosity, offers more uniform particle distribution, and allows a higher filler load. Indeed, the real innovation with nano-fillers consists on their ability to increase the load of the inorganic phase, which represents an 80% in weight of the filler load compared to the 50% wt. of the micro-filled.

Moreover, recent applications of nanotechnology have also been used to increase the bonding performance of several enamel-dentin adhesive systems. Indeed, the principal concern in adhesive dentistry is to achieve durable materials that bond to dental tissues in order to increase the longevity of the restorations. Current adhesive systems allow clinicians to restore tooth structure minimizing cavity preparation, by simply providing an immediate bond of different restorative materials (16). However, the hybrid layer, or rather the bonded interface between dentin and resin, can result unstable and fail over time, thus leading to marginal leakage and poor retention of the restoration. Several strategies to reduce the degradation of the resin-dentin bonded interface have been proposed (115), as following: Application of factors inhibiting the enzymes responsible for the hybrid layer degradation; agents cross-linking dentin matrix, that improve dentin biomechanical properties; modifications in the chemical formulation of adhesives components to manage water adsorption, thus reducing collagen



degradation; addition of P and Ca ion-releasing materials which imitate the natural dentin mineralization using nano-apatite crystallites to fill the demineralized regions (16).

In spite of the above-mentioned nanotechnological applications in restorative dentistry, polymerization shrinkage stress (PSS) still represents the most significant problem. PSS decreases the adhesive bond strength, which leads to negative clinical implications such as post-operative sensitivity, enamel cracks, and marginal gap formation, which ultimately conducts to the development of secondary caries. Several factors such as the composite formulation, elastic modulus of the material and light irradiance delivered by the curing unit, can affect the polymerization shrinkage stress (14). Several studies (40,170) have confirmed that the cavity configuration (C-factor), known as the ratio between bonded and unbonded surfaces of the composite restoration, plays a crucial role in PSS. Cavities with a high C-factor and those with large dimensions have increased PSS, and consequently reduced bond strength, thus initiating a potentially deleterious effect on marginal integrity and gap formation (41). In order to limit PSS, incremental filling techniques were introduced, whereby resin composite layers are built-up in increments and then cured separately (110). This procedure has long been accepted as a standard technique mainly to guarantee adequate depth of cure and reduced PSS. Nevertheless, the incremental filling technique causes problems such as void formation among layers and is time-consuming, thus increasing the cost associated to the restoration placement (14,60,68).

3.1 Bulk-fill composites

A new generation of RBC with a new monomer content and enhanced curing properties, known as bulk-fill composites (BFC), have been recently introduced to overcome the limitations



of the incremental filling technique. BFC have gained success among clinicians for their user-friendly application, especially for posterior restorations (105). BFC can be cured in a maximal increment thickness of 4 mm with limited shrinkage, thus allowing clinicians to fill the cavity in one single step with a satisfactory cavity adaptation and with the consequence of simplifying the procedure and decreasing the chair-time needed to complete the restoration (121). Depending on their consistency, BFC are classified into high and low viscosity. The physical properties of high-viscosity BFC, also known as packable, allow to finalize the restoration without further application of a final occlusal capping (121). On the other hand, low-viscosity BFC, also known as flowable or flow, easily adapt to any surface irregularities present on the cavity floor, in order to obtain a standardized cavity, especially in clinical cases where there is less accessibility. The low filler content of low-viscosity BFCs renders the surface less wear resistant. Therefore, a final occlusal capping with a high-viscosity BFC is required with their usage (170). Conversely, high-viscosity BFC do not need a final capping, hence, they can be used alone to fill a cavity up to the occlusal surface.

Despite the clinical and scientific evidence of the advantageous properties of BFC (67), more investigations on their mechanical and clinical performance are needed, particularly with regards to PSS and the internal adaptation.

The measurement of the shrinkage in a cavity is technically challenging; however, several in vitro methods have been described in the literature to analyze the PSS effect (87). The most frequently used method to examine internal adaptation, which evaluates the interface between the resin composite and the dental substrate, is the dye penetration using basic fuchsin, methylene blue, erythrosine, silver nitrate, or radioactive markers (57). However, in utilizing this technique, the samples need to be sectioned, which limits further material characterization and may lead to false interfacial leakage. Other methods include optical instruments, fiber-optic sensing system, mercury dilatometer, electromagnetic balance and video imaging (56). The



internal adaptation can also be evaluated by scanning electron microscope after sectioning. This method is very sensitive, but has important limitations regarding quantitative assessment, which is based on the operator's visual evaluation and does not represent, being two-dimensional (2D), the entire gap volumes. To overcome these drawbacks, X-ray microtomography (μ-CT) has recently been introduced to evaluate both PSS and the internal adaptation of RBC (92).

3.2 X-ray microtomography

Since 1895, the x-ray imaging represents a fundamental diagnostic tool to observe the inside of the human body in a noninvasive way (183). As an evolution of the x-ray radiography, the computed tomography (CT), developed in 1972 by Godfrey Newbold Hounsfield, was the first method able to produce images of trans-axial planes through the human body (slices), without being biased by superimposition of distinct anatomical structures. Nowadays, the enormous technological application, regard higher resolution setups, developed the x-ray computed micro-tomography (μ-CT) system that is able to reach a micrometric spatial resolution on centimeter-sized samples and a sub-micrometer spatial resolution on samples at the scale of 1–2 mm (nano-tomography) (184).

During a μ-CT scan, the object is illuminated by x-rays beam that, after passing through the object, is collected by a detector (Figure 2).

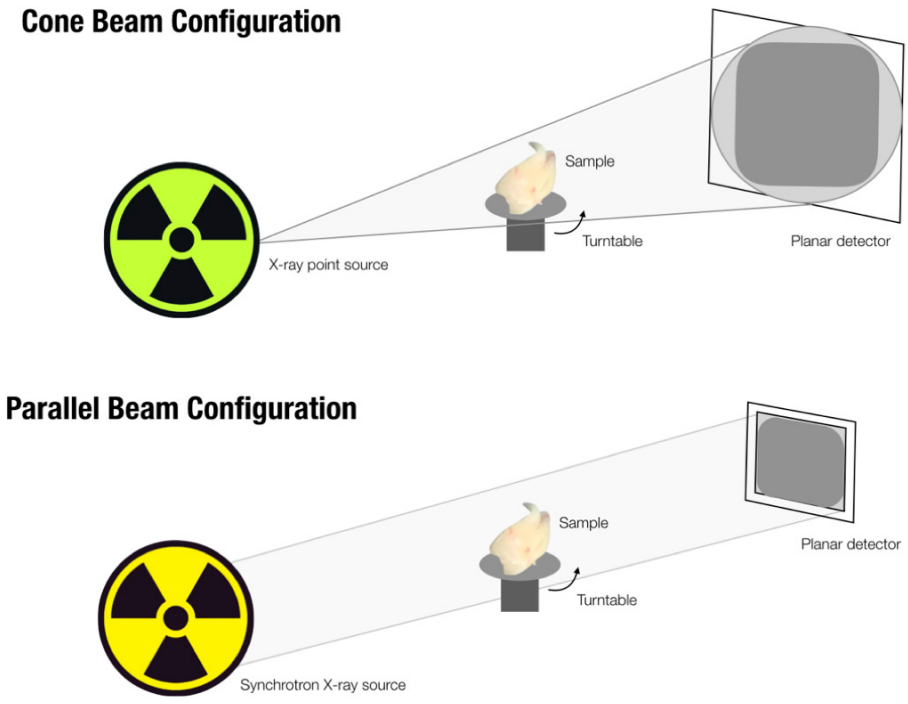

Figure 2. Illustration of different beam configuration geometries,

A series of bi-dimensional projections are taken, usually at a fixed angular increment. For in vitro studies, the source and detector are fixed, and the sample is placed on a rotating stage. Then, starting from the totality of projections, are reconstructed the 3D tomographic slices, or rather the images of the internal structure of the object in a plane parallel to the beam propagation direction. The principle on which relies the image formation can be either absorption or phase-contrast effect. In conventional x-ray imaging, the image formation is based on the differences in x-ray absorption of the sample details (185). For example, dense structures like bones absorb more than lighter elements comprising soft tissues; therefore, μ-CT is based on the mapping of the linear attenuation coefficient of x-ray crossing the investigated sample, and the attenuation depends upon the composition and density of the object. When structures with a similar absorption property are observed, the overall image contrast may be insufficient to obtain a distinct meaningful image. However, as x-rays are electromagnetic waves, not only their absorption but also the relative phase shifts can provide



information about an object. So, phase contrast plays an essential role to obtain sufficient image contrast for x-ray imaging of soft biological tissues and biological samples, where conventional absorption radiography fails (119,120).

The μ-CT is similar to conventional computer tomography usually employed in medical diagnosis and industrial applied research, and is capable of achieving a spatial resolution of up to 0.3 μm, i.e. about three orders of magnitude higher (149). Due to the penetrating capacity of x-rays, μ-CT allows clinicians to accurately evaluate the restoration without destroying the samples, allowing to examine its internal aspects, irrespective of a sample's shape or dimensions (72). Indeed, the μ-CT data can be reconstructed into three-dimensions (3D) to obtain, in a non-invasive and non-destructive way, qualitatively and quantitatively valuable information (46), for instance providing accurate information on the gap formation caused by air bubbles, regardless of the object shape or its position (92). In previous studies focused on dental restorations, a resolution of a few micrometers has been used in order to analyze the structures at dentin-adhesive-composite interfaces before and after mechanical loading and to evaluate resin composite volume and 3D marginal adaptation before and after polymerization (2,46).

The μ-CT has been also shown to be a reliable and effective investigative tool for determining the precise volume and position of RBC in cavities used as models. Moreover, because of its 3D imaging capabilities, the μ-CT represents the preferred non-destructive method to quantify microleakage, obtaining accurate results on PSS, in agreement with those obtained by dye penetration (162). Furthermore, μ-CT data could also enable a better understanding of PSS and its dependency to the elasticity modulus of materials (149). The accuracy of the μ-CT 3D imaging has been also shown to allow the visualization of bubbles, which cannot be rendered by conventional methods, such as replica techniques (149). Therefore, μ-CT can be considered an accurate method to further evaluate BFC, by testing new filling techniques and cavity



preparations in an effort to obtain a more favorable C-factor, avoiding PSS and marginal gap formation.

For this reason, thanks to the collaboration of the research team of the Physics Section of our Department, headed by Prof. Alessandra Giuliani, an interesting study regarding μ-CT has been realized [Paper VII]. This research aimed to compare two different bulk-filling techniques, evaluating the internal and external adaptation of class II RBC restorations, by analyzing the gap formation using μ-CT and SEM coupled with energy-dispersive X-ray spectroscopy (EDS). The obtained results highlighted that the two filling techniques do not show significant differences regarding the internal and external marginal adaptation of the restoration. Therefore, to achieve a successful restoration, the clinician could be advised to restore a class II cavity (i.e. when caries lesion is located on a proximal surface of a posterior tooth) using a single increment bulk-filling technique, thus treating it as a class I cavity (i.e. when caries lesion is placed in the pits and fissures of the occlusal surface of a tooth).

In addition to the evaluation of different filling techniques, a new modelling procedure using the advantageous properties of BFCs, has been proposed to simplify the RBC restoration, achieving an optimal final result. The novel technique, called “Essential lines”, reproduces the occlusal morphology of the posterior teeth when executing direct posterior RBC restorations. The concept behind each step of the Essential lines technique as well as some clinical case examples have been realized thanks to the fruitful collaboration with Dr. Giuseppe Chiodera, leading to a final original article already accepted and in press, in an internationally renowned clinical magazine [Paper VIII].



3.3 Finishing and polishing systems

In order to preserve and correctly mimic the physiological topographies of the tooth to be restored, it is critical to reproduce adequate surface features of the restorative materials, trying to reach the same surface roughness, gloss, and color stability of the natural tooth (147). Nowadays, nano-hybrid and nano-filled RBC are considered universal materials suitable for the restoration of both anterior and posterior teeth, due to their excellent aesthetic properties (10). As noted above, the type and size of the filler particles influence the physical and mechanical characteristics of the material and protect the organic matrix against the force applied to the direct restoration, having a direct influence on the surface properties of the composite such as the roughness and surface gloss (86). Indeed, the nano-particles are less susceptible to detaching after finishing and polishing procedures (53). Most RBC restorations require finishing after placement in order to remove overhangs and excess of RBC, as well as properly re-establish the occlusion and the correct morphology (3,32).

Finishing refers to the gross reduction of material excesses to achieve the proper morphology and optimal function. It is achieved by using either fine and extra-fine diamond burs, or multi-fluted tungsten carbide burs (43,79,123). However, the finishing step increases the surface roughness above the acceptable threshold, thus, polishing step is needed to decrease the surface roughness to satisfactory values (10). Polishing is defined as the reduction of roughness and scratches which were created whilst finishing the RBC (43). Due to the importance of the aesthetic results, it would be useful understanding better the finishing and polishing procedures and evaluate their effect on the RBC, in particular in terms of surface roughness and gloss retention.

The surface roughness and gloss retention represent the most important features affecting the appearance and the clinical success of dental restorations. Surface roughness has an influence on the amount of plaque accumulation in the RBC surfaces and plays an important role in the



aesthetics of the restoration (123). Although the patients can detect a surface roughness greater than 0.50 μm with their tongues, a surface roughness threshold of below 0.2 μm is necessary to prevent the risk of biofilm adhesion and maturation, linked with gingival inflammation and surface staining (10,85). In the scientific literature, the smoothest surface is obtained when RBC polymerize against a Mylar strip without subsequent finishing or polishing (6). However, such flat surface gives an uneven geometrical light distribution reflected by the surface.

The gloss retention is influenced by the surface roughness: The light reflected off every angle of the surface as well as the particle size of the material, being associated with the amount of light reflected by the material itself (10). Therefore, a smoother surface has a higher gloss, indicating superior clinical durability and ideal aesthetic appearance, thus inducing optimal optical compatibility between RBC and the natural tooth enamel (101).

Due to the importance of such treatments, clinicians can choose a wide variety of finishing and polishing instruments. However, there are different finishing and polishing systems available on the market (4), including flame-shaped polishers in several sizes and brush for occlusal surfaces; cups for proximal, buccal and lingual surfaces of restorations, discs for anterior restorations as well as the proximal surfaces of posterior ones (69). Several studies have demonstrated that multi-step aluminum oxide discs produced the best surface smoothness (45,182). However, because of the shape of such discs, they provoke a flat surface, and especially in posterior restorations, do not allow to create the right anatomy. In addition, other finishing and polishing systems exist to treat posterior and anterior areas, by means of multi-fluted burs, abrasives and polishing pastes used in one, two or multi-steps. In the last years, many attempts focused on optimizing the finishing and polishing instruments (186).

Several in vitro investigations were carried out in order to evaluate the effect of different finishing and polishing procedures, to identify the technique that produces the smoothest surface



possible and to identify which procedure increases the gloss values and allows to obtain an acceptable morphology of nanocomposites (5,87,97). However, no consensus on this topic has been reached yet.

In this regard, the behavior of four finishing and polishing systems on RBC was evaluated in the Department of Clinical Sciences and Stomatology [Paper IX]. The results showed that roughness and gloss surface of RBC depend on the finishing and polishing systems used. Moreover, a polishing system based on a diamond paste can offer to the clinician the possibility of achieving high gloss values, as well as adequate smooth and mirror-like surfaces, thus making the base for properly finalizing nanocomposites restorations.

Furthermore, although there are many articles on the effects of polishing on traditional RBC [8, 13], no detailed studies exist concerning the timing effects of the new polishing systems on the properties of BFC. Therefore, a further work was conducted in order to improve the knowledge on the effects of immediate and delayed polishing on packable and flowable BFC [Paper X]. Our findings showed that in the case of flowable BFC, is recommended waiting 24 hours before polishing, because the surface is more regular in delayed than in immediate polished samples. On the other hand, the immediate polishing of restorations performed using packable BFC could definitively be recommended, since its properties were increased and a smooth and regular occlusal surface was also observed. Thus, clinicians may always perform and finalize their restorations in one single appointment, saving time, without compromising the good physical properties of these BFC.

3.3 Resin luting agents

The development of modern materials for adhesive dentistry and the advent of highly aesthetic restorative materials have changed the cementation procedure of indirect



restorations. As noted before, a strong adhesion of the restoration to the residual dental tissues allows the clinician to preserve as much as healthy tissue as possible (39,124), performing a non-retentive preparation, regardless to the shape and the materials choice for the indirect restoration (95). Indeed, preparation principles based on minimally invasive dentistry and resin-based luting agents are becoming the gold standard for realizing successful indirect adhesive restorations (39,172). Supported by the recent scientific literature, there is no consensus on which luting material should be used to cement. The luting agents are intermediates between the tooth substrate, with or without a bonding agent, and the indirect restoration. The luting agents can be divided in the following families: based on the type of activation of their own monomers, into light, self or dual cured; and based on the consistency of their formulation, into low viscosity luting agents (LVLA) and high viscosity luting agents (HVLA) (91). LVLA consist of resin cements and flowable resin-based composites. On the other hand, HVLA consist of traditional and bulk-fill RBC. Because during clinical application the thickness of the indirect restoration can reduce the amount of curing light that reaches the resin luting agent, then, the type of curing becomes a fundamental factor for the success of the restoration [6, 7]. For example, with a thick inlay/overlay restoration or with deep cavities, clinicians are uncertain whether the resin luting agent has been properly polymerized. Therefore, improving the knowledge on polymerization kinetics could be relevant, particularly during the cementation of an indirect restoration. Moreover, the management and removal of the material excesses depend mainly by their consistency (48,150). During the cementation of indirect restoration, the removal of the cement excesses from the preparation margins remains a crucial step for the survival rate of the restoration, mainly when using LVLA. Indeed, residual resin material close to the gingival margin can provoke inflammation and change the survival rate of the restorations (175). Indeed, some studies highlighted the detrimental impact on the periodontal tissue of the residual luting agent, resulting in accumulation of plaque and oral pathogens,



which can lead to periodontal disease (23,175). Consequently, it is fundamental the complete removal of excess luting agent from subgingival areas, especially when preparation margins are in close proximity of the CEJ. Indeed, several works investigated that undetected luting agent remnant excesses were more likely to be found when the restorations present deeper subgingival margins (103,108,175). For this reason, during the luting phase, some clinicians adopt a step curing technique to fix the indirect restoration, allowing to remove easily the soft material excess over the indirect restoration.

Since no scientific articles or evidences have claimed the chemical stability after this kind of "step luting" procedure, our in vitro research was carried out to deepen the knowledge on polymerization kinetics, particularly during the cementation of an indirect restoration [Paper XI]. Therefore, analyzing two different light-cured protocols and since they did not present statistical difference, we can conclude that the clinician can safely use the tested step of cementing protocol (5 s + 40 s) to efficiently lute the indirect restoration, simplifying the removal of cement excesses, in particular in the interdental spaces.

Innovative kits and techniques are completely revolutionizing the world of dental care and treatment. Thanks to the advanced nanotechnological tools and the highly performing, aesthetic and durable biomaterials, restorative dentistry can still be considered one the most useful methods in rehabilitating patient's smile, by using either direct or indirect restorations.



Papers VII-XI

VII. Tosco V, Vitiello F, Furlani M, Gatto M.L, Monterubbianesi R, Giuliani A, Orsini G, Putignano A (2021) Microleakage Analysis of Different Bulk-Filling Techniques for Class II Restorations: μ-CT, SEM and EDS Evaluations. Materials, 14, 31. https://doi.org/10.3390/ma14010031

VIII. Chiodera G, Orsini G, Tosco V, Monterubbianesi R, Manauta J, Devoto W, Putignano A (2021) Essential Lines: a simplified filling and modelling technique for direct posterior composite restorations. Int. J. Esthet. Dent. 202;16:168–84.

IX. Tosco V, Monterubbianesi R, Orilisi G, Procaccini M, Grandini S, Putignano A and Orsini G (2020) Effect of four different finishing and polishing systems on resin composites: roughness surface and gloss retention evaluations. Minerva Stomatol.;69(4):207-214. doi: 10.23736/S0026-4970.19.04310-3. Epub 2019 Oct 15. PMID: 31633320.

X. Monterubbianesi R, Tosco V, Sabbatini S, Orilisi G, Conti C, Özcan M, Orsini G, Putignano A (2020) How Can Different Polishing Timing Influence Methacrylate and Dimethacrylate Bulk Fill Composites? Evaluation of Chemical and Physical Properties. BioMed Res. Int, Article ID 1965818, 8 pages, 2020. https://doi.org/10.1155/2020/1965818

XI. Tosco V, Monterubbianesi R, Orilisi G, Sabbatini S, Conti C, Özcan M, Putignano A, Orsini G (2020) Comparison of two curing protocols during adhesive cementation: can the step luting technique supersede the traditional one? Odontology. doi: 10.1007/s10266-020-00558-0. Epub ahead of print. PMID: 33128650.



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Article

Microleakage Analysis of Different Bulk-Filling Techniques for Class II Restorations: μ-CT, SEM and EDS Evaluations

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Abstract: This study aimed to compare two different bulk-filling techniques, evaluating the internal and external adaptation of class II resin-composite restorations, by analysing the gap formation using microcomputed tomography (μ-CT) and scanning electronic microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDS). Two standardized mesio/disto-occlusal (MO/DO) cavities were prepared in eight extracted human third molars that were divided, according to the filling technique used, in the following two groups (n = 4): BG (Bulk&Go group) and BT (Bulk Traditional group). After universal bonding application, followed by the light curing, all teeth were restored using a bulk-fill composite. Specimens were scanned with μ-CT to evaluate 3D interfacial gaps. Acquired μ-CT data were analysed to quantify the gap formation. Complementary information to the μ-CT analysis were obtained by SEM. Thereafter, the chemical composition of tooth–restoration interface was analysed using EDS. The μ-CT analysis revealed gaps formation at the tooth–restoration interface for both the BG and BT groups, while within the restoration, only in the BT group there was evidence of microleakage formation. The scanning electron micrographs of both groups showed that the external marginal integrity of the restoration was preserved, while EDS showed the three different structures (tooth surface, adhesive layer and resin composite) of the tooth–restoration interface, highlighting the absence of gap formation. In both BG and BT, the two filling techniques did not show significant differences regarding the internal and external marginal adaptation of the restoration. To achieve a successful restoration, the clinician could be advised to restore a class II cavity using a single increment bulk-filling technique (BG), thus treating it as a class I cavity.

Keywords: resin composite; dental restoration; scanning electron microscopy; microcomputed tomography; X-ray spectroscopy



Citation: Tosco, V.; Vitiello, F.; Furlani, M.; Gatto, M.L.; Monterubbianesi, R.; Giuliani, A.; Orsini, G.; Putignano, A. Microleakage Analysis of Different Bulk-Filling Techniques for Class II Restorations: μ-CT, SEM and EDS Evaluations. *Materials* **2021**, *14*, 31. https://dx.doi.org/10.3390/ma14010031

Received: 27 November 2020
Accepted: 21 December 2020
Published: 23 December 2020

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1. Introduction

Dental caries represents one of the most prevalent diseases in the world and remains the main challenge for the clinician [1]. It can be classified by location and extent of the lesions produced by the demineralization and, according to the G.V. Black classification system, a lesion placed in the pits and fissures (grooves) of the occlusal (biting) surface of a tooth is considered a class I lesion, whereas a lesion located on a proximal surface of a posterior tooth is considered a class II lesion [2]. The standard treatment for a carious lesion consists of the removal of the infected tooth structure, followed by its restoration using a dental material. Resin composites have become the gold standard in restorative dentistry for their primary role in guaranteeing excellent aesthetics and function [3]. Despite the ongoing research to obtain sophisticated composites with optimal characteristics, these materials may fail predominantly due to their occlusal wear or the



insurgence of a secondary caries, due to microleakage that potentially provides loss of marginal integrity [4,5]. Indeed, the polymerization shrinkage stress (PSS) and the deriving microleakage still represents the most significant complication after composite restoration. PSS decreases the adhesive bond strength, which leads to negative clinical implications such as post-operative sensitivity, enamel cracks, and marginal gap formation, ultimately leading to the development of secondary caries [6,7]. Moreover, several studies [8] have confirmed that the cavity configuration (C-factor), known as the ratio between bonded and unbonded surfaces of the composite restoration, plays a crucial role in PSS. The C-factor is an important variable that must be considered when placing composite materials inside the cavity [9]. Cavities with a high C-factor and those with large dimensions have increased PSS, and consequently reduced bond strength. Indeed, especially in large class II cavities with dentin and cementum margins [10,11], despite a lower C-factor compared to class I cavities, a potentially deleterious effect on marginal integrity and gap formation can occur [8]. In order to limit PSS, incremental filling techniques were introduced, whereby resin composite layers are built-up in increments and then cured separately [12]. While the incremental filling technique reduces PSS, it also causes problems such as voids formation among layers and extends the time required to place restorations [13]. In the attempt to decrease the microleakage and shorten the working time, a new generation of resin composites with a novel monomer content and enhanced curing properties, known as bulk-fill composites (BFC), have been recently introduced. BFC have gained success among clinicians for their user-friendly application, especially for posterior restorations [14]. BFC can be cured in a maximal increment thickness of 4 mm with limited shrinkage, thus allowing clinicians to fill the cavity in one single step. This technique, called bulk-fill technique, lets to simplify the procedure with a satisfactory cavity adaptation, allowing to complete the restoration decreasing the chair-time needed, also assuring adequate physical surficial properties [15,16]. Although there are clear clinical and scientific evidences of the advantageous properties of BFC [12,17], more investigations of their mechanical and clinical performances are needed, particularly with regards to PSS and the internal adaptation of the restoration.

The measurement of the shrinkage in a cavity is technically challenging; thus, several in vitro methods have been described in the scientific literature to analyse the PSS effect [18]. The evaluation of leakage in class II restorations is a common procedure of analysing the long-term stability of adhesive and composite resin systems [19]. The most frequently used method to examine the internal adaptation is the dye penetration, which evaluates the interface between the resin composite and the dental substrate, using basic fuchsin, methylene blue, erythrosine, silver nitrate, or radioactive markers [20]. However, in performing this technique, the samples need to be sectioned, which obviously limits further material characterization and may lead to false interfacial leakage. The internal adaptation can be also evaluated by scanning electron microscope (SEM) after sectioning, obtaining a qualitative evaluation of the surface morphology. Moreover, the energy dispersive X-ray spectroscopy (EDS) is used to investigate the chemical composition of the tooth–restoration interface. Therefore, the effort of coupling these analytical techniques represents a valuable tool to obtain a comprehensive overview of the morphological different structure of tooth/material interface. However, this method is very sensitive, and has several limitations concerning the quantitative assessment, which is based on the operator's visual evaluation and does not represent, being two-dimensional (2D), the entire gap volumes.

To overcome all these drawbacks, X-ray micro-computed tomography (μ-CT) has recently been introduced to evaluate both PSS and the internal and external adaptation of resin composites [21–23]. Due to the penetrating capacity of X-rays, μ-CT allows clinicians to accurately analyse the restoration without sectioning the samples. Furthermore, it allows to examine internal aspects, irrespective of a sample's shape or dimensions [24]. Indeed, the μ-CT data can be reconstructed into three-dimensions (3D) to obtain, in a non-invasive and



non-destructive way, qualitatively and quantitatively valuable information [25], for instance providing accurate information on the gap formation caused by air bubbles [21].

Therefore, the aim of this study was to evaluate and quantify marginal gap formation, air bubbles, and microleakage at the tooth–restoration interface, analysing two different bulk-filling techniques in class II restoration, by means of an innovative experimental protocol based on the combined use of μ-CT, SEM and EDS.

2. Materials and Methods

2.1. Specimen Preparation

For this study eight sound extracted third molar were collected, from subjects aged between 18 and 30 years. Teeth were surgically extracted for orthodontic reasons at the Section of Stomatology of DISCO Department, Polytechnic University of Marche, Ancona, Italy. According to the Local Ethic Committee guidelines and the 1964 Helsinki declaration, informed consent was obtained from the subjects that were aware that their hard-dental tissues, as discard of the surgical procedures, would be used for research purpose. The inclusion criteria were intact crowns, free of caries, hypoplastic defects, cracks and restorations. Scaler and a hand-scaling instruments were used for surface debridement. Two separated standardized class II slot cavities, mesio-occlusal (MO) and disto-occlusal (DO) were prepared in each tooth (for a total of 16 cavities in 8 teeth) using diamond burs (206/845, Komet Dental, Brasseler GmbH and Co., Lemgo, Germany) on high-speed handpiece (Kavo, Biberach, Germany), under water cooling. The bur was replaced after every five cavities preparations in order to maintain cutting efficiency. The dimensions of the cavities were standardized: Buccolingual width of 4 mm, axial wall of 3 mm from the proximal surface. The cervical margin was placed 1 mm below the CEJ (cemento-enamel junction) (Figure 1a).

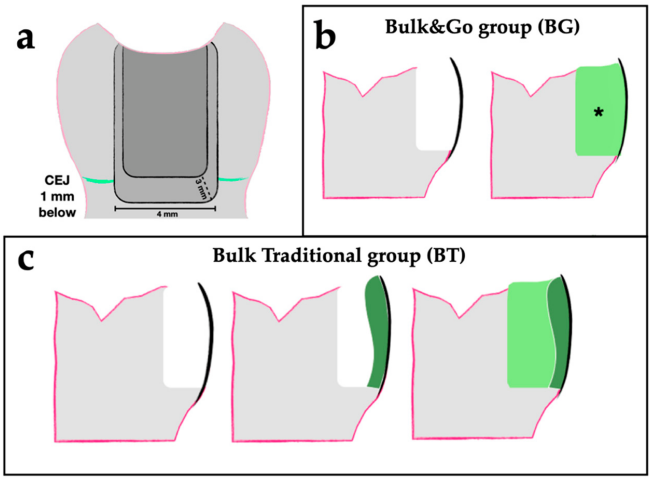

Figure 1. Cavity design and samples preparation. Panel (**a**) represents the stylized cavity preparation of the samples from the mesial view. Panel (**b**) shows the Bulk&Go filling technique: After cavity preparation and automatrix application, only one single increment of BFC was set directly into the cavity, without performing the proximal wall (asterisk). Panel (**c**) displays the Bulk Traditional filling technique: After cavity preparation and automatrix application, the proximal wall was firstly restored, using a bulk-fill composites (BFC), transforming class II into class I (dark green), then the residual cavity was filled using one single increment of BFC (light green).

All teeth were stored in 0.5% *w*/*w* chloramine solution (NH_2Cl) at room temperature (25 °C) before and after preparation procedures. After cavities preparation, two different



groups including 4 teeth each (n = 8 cavities per group) were formed, according to the filling technique:

- Bulk&Go group (BG): Each class II cavity was filled using the bulk fill technique (one BFC single increment to the occlusal surface) (Figure 1b);
- Bulk Traditional group (BT): To begin, the proximal wall was restored in order to transform class II into class I, then the residual cavity was filled using one single increment of BFC (Figure 1c).

All samples, in both groups, were restored using the high viscosity BFC, Filtek One Bulk Fill Restorative (3M ESPE, St. Paul, MN, USA; Table 1).

Table 1. Composition of the BFC material used in this study.

Material	Manufacturer	Composition
Filtek One Bulk Fill Restorative	3M ESPE, St. Paul, MN, USA	Fillers: Non-agglomerated nanosilica of 20 nm filler size and agglomerated zirconia/silica nanocluster with a size of 5–20 nm. The filler loading was 76.5 wt.% (58.5% by volume). Organic matrix: Bisphenol-A glycidyl dimethacrylate (Bis-GMA) (1–10 wt.%), urethane dimethacrylate (UDMA) (10–20 wt%), triethylene glycol dimethacrylate (TEGDMA) (<1 wt.%), bisphenol A polyethylene glycol diether dimethacrylate (Bis-EMA)-6 (1–10 wt.%), in addition to addition–fragmentation monomer (AFM), aromatic urethane dimethacrylate (AUDMA), and 1,12-dodecane dimethacrylate (DDDMA).

Firstly, a circumferential metal matrix (Automatrix MT, Dentsply, Milford, DE, USA) was placed around each tooth, adjusted and fixed around the cavity for a good adaptation. Afterwards, 37% phosphoric acid (3M Scotchbond Etching Liquid, Saint Paul, MN, USA) was applied to the enamel for 30 s (selective enamel etching) and rinsed for 30 s. Specimens were gently air-dried with an air syringe, preventing dehydrating. After that, all groups were treated with Scotchbond Universal bonding, (3M Oral Care, Saint Paul, MN, USA), and "air blowing" for 20 s was accomplished, according to manufacturer's instructions. Polymerization was carried out by means of the curing lamp LED Elipar (Elipar S10, 3M ESPE, St. Paul, MN, USA) for 40 s with a light irradiance of 1200 mW/cm^2. The composite material was compacted and adapted, removing the excesses, using the LM-Arte Condensa instrument (LM-Instruments Oy, Parainen, Finland) [26]. Once the restoration was completed, the occlusal surface was finished and polished using abrasive discs (Sof-Lex Discs Coarse and Medium, 3M ESPE, St. Paul, MN, USA), rubber cups (Opti1Step, Kerr Dental, CA, USA), and brushes (Occlubrush Regular, KerrHawe, Boggio, Switzerland); while the interproximal surface was finished and polished using strips (OptiStrip, KerrHawe, Boggio, Switzerland).

2.2. Micro-Computed Tomography Analysis

After the restoration, each sample was individually scanned by means of a micro-computed tomography system (μ-CT) Bruker SkyScan 1174 (SkyScan-Bruker, Antwerp, Belgium), installed at the Laboratories of the Research Center and Microscopy Service of Nanostructures (CISMiN), at the Polytechnic University of Marche, Ancona, Italy. Projections settings were as follows: Acceleration voltage 50 kV; beam current 800 μA; aluminium filter of 1 mm; pixel size 9.5 μm and rotation 180° in 0.2° step with an exposure time of 10 s per projection. The average scan time was 5 h. The total number of reconstructed sections for each specimen was approximately 950, obtaining axial information of approximately 9 mm tooth thickness. Tooth projections were converted to cross-sectional slices by NRecon software (Version 1.6.10.2, Bruker, Billerica, MA, USA) with the following correction



settings: Ring artefacts (8.0); smoothing (6.0); beam hardening (70%) and proper misalignment compensation. The 3D analysis software VGA Studio MAX (Volume Graphics, vers. 1.2.1, Heidelberg, Germany) was used to perform image analysis and assessment of gap formation within the tooth–restoration interface.

In particular, to evaluate internal adaptation, the *Defect Analysis Tool* of VGA Studio MAX was used. The operation tool was designed to process voxel datasets for internal defects. The parameters quantified were: The number of defects detected at the interface and within the restoration (Nr of defects); the total volume of defects (DV, expressed in μm^3), the specific surface, i.e., the ratio between the surface and the volume of the defects (DS/DV, expressed in μm^{-1}); the mean thickness of the void formation (Mean Thickness, expressed in μm); the volume of mineralized materials (enamel and dentin) and composite (MV, expressed in μm^3); and the ratio of the latter value to the total volume of the defect (DV/MV, expressed in percentage).

The CTAnalyser software (vers. 1.17.7.2, Bruker Billerica, MA, USA) was employed to create 3D model considering a stack of 200 cross-sectional slices within the defects of interest. 3D reconstructions of enamel, dentin, restorations and gaps, were jointed in a unique colored tooth model by CTvol software (vers. 2.0, Bruker Billerica, MA, USA).

2.3. Scanning Electron Microscopy and Energy-Dispersive X-ray Spectroscopy Analysis

Thereafter, the specimens were fixed in metallic stubs, sputter-coated with gold and observed by a scanning electron microscope (SEM). Scanning electron micrographs were performed for evaluating the presence of gaps and misfits pointed out at the μ-CT (SkyScan-Bruker, Antwerp, Belgium). The images were acquired with a ZEISS SUPRA 40 (CISMiN, Univpm) (ZEISS, Oberkochen, Germany). Firstly, external mesial and distal walls of class II restoration were evaluated at different magnifications. After that, all samples were sectioned according to the axial plane with a diamond blade saw (Buehler IsoMet, Buehler, Manassas, VA, USA) to visualize the internal marginal adaptation and the presence of internal defects of the restoration (at different magnification). For SEM analysis, the surfaces of the sectioned samples were finished and polished with a lapping machine (Buehler Metaserv, Buehler, Lake Bluff, IL, USA) using 600 and 800 grit silicon carbide abrasive papers. The chemical characterization of the tooth–restoration interface was carried out by energy dispersive X-ray spectroscopy (EDS) technique (Bruker, Berlin, Germany), performed by EDAX Element microanalysis. The same samples were used for SEM and EDS. The operating parameters were: 15 mm working distance, 25 kV accelerating voltage at 3000× magnification. The concentrations by weight (%) of the several chemical elements were evaluated.

2.4. Statistical Analysis

The statistical analysis of the morphometric data obtained from the μ-CT was performed using the software package SigmaStat 3.5 (Systat, San Jose, CA, USA). T-tests were performed between the BG and the BT groups on the following parameters: Nr of Defects, DV, DS/DV, Mean Thickness, MV, DV/MV. The statistical significance was analysed at three levels: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. All values were expressed as mean and standard deviations.

The statistical power of the experiment was calculated by means of a Post-hoc Power (PhP) Calculator (ClinCalc.com) (Kane SP. Sample Size Calculator. ClinCalc: https://clincalc.com/stats/samplesize.aspx.), setting Continuous Endpoint, Two Independent Sample Study, and $\alpha = 0.05$.

3. Results

The quantification of parameters obtained by internal microleakage analyses using μ-CT on both BG and BT groups are shown in Table 2. Means and standard deviations (m ± SD) for all parameters analysed in BG and BT groups were reported.

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Table 2. Means ± Standard Deviation o (m ± SD) of data obtained by the Defect Analysis Tool of BG and BT groups. Nr of defects (Number of defects), DV (Defect Volume), DS/DV (Defect Surface/Defect Volume), MV (Materials Volume), DV/MV (Defect Volume/Materials Volume).

Group	Nr of Defect	DV ($\times 10^9$, μm^3)	DS/DV (μm^{-1})	Mean Thickness (μm)	MV ($\times 10^9$, μm^3)	DV/MV (%)
BG	21.0 ± 1.0	0.19 ± 0.18	0.085 ± 0.016	24.250 ± 4.969	714 ± 72.7	0.028 ± 0.034
BT	21.5 ± 11.4	2.08 ± 1.09	0.025 ± 0.015	112.032 ± 54.202	829 ± 131	0.268 ± 0.163

The results obtained of various parameters in both groups is represented through the graphs in Figure 2, analysing the data reported in Table 2.

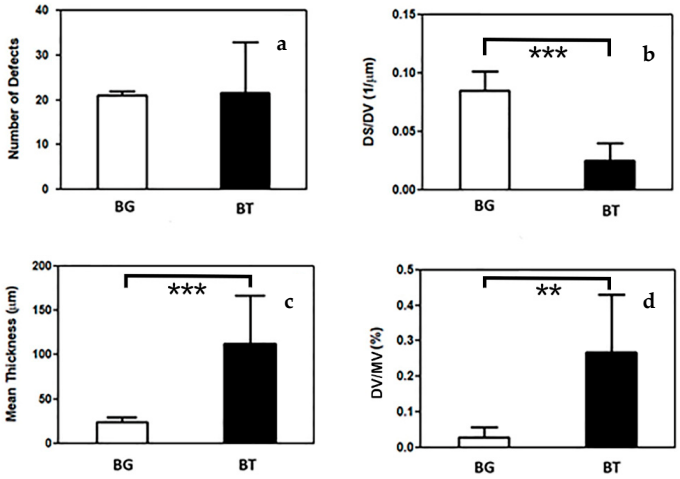

Figure 2. Results obtained by the Defect Analysis Tool, which compared BG and BT groups. As resulting from Table 2, (**a**–**d**) show the number of defects (**a**), DS/DV (**b**), mean thickness (**c**) and DV/MV (**d**). Three levels of statistical significance were considered: $p < 0.01$ **, $p < 0.001$ ***.

In general, we observed higher standard deviations in the BT than in the BG group for almost all the considered parameters. Moreover, significantly higher specific surface was calculated in the BG group ($p < 0.001$; PhP = 100%) and significantly lower mean thickness ($p < 0.001$; PhP = 99.6%) and DV/MV ($p = 0.001$; PhP = 98.3%) than in the BT group.

SEM analysis displayed the different surface morphology of each sample. Figure 3 displayed the scanning electron micrographs representative of BG and BT samples. Scanning electron micrograph of BG showed that the external marginal integrity of the restoration was preserved (see arrows in Figure 3a). Also, for BT no external gap formation was observed (arrows in Figure 3d).

The combined analysis of SEM and μ-CT data well displayed internal defects, as shown in virtual transversal sections of the restorations performed in the two BG and BT groups (Figures 4 and 5).

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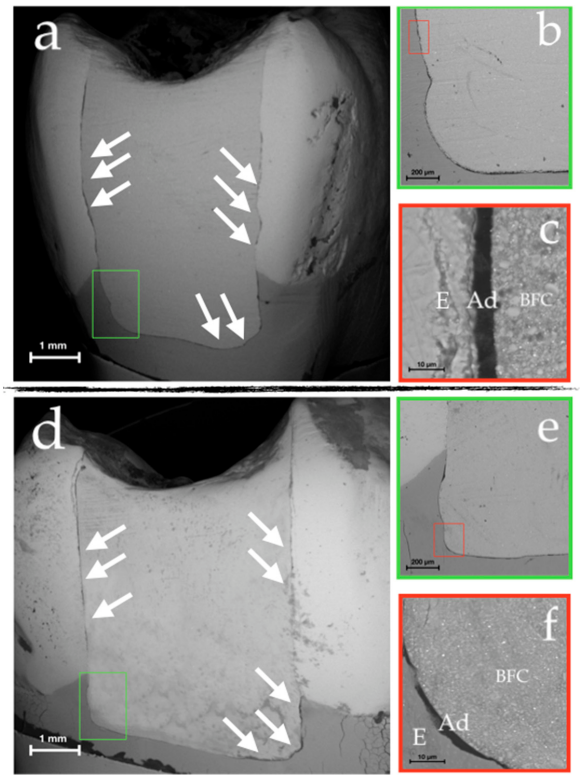

Figure 3. This scanning electron micrographs display a representative mesial view of class II restoration of BG (Bulk&Go group) and BT (Bulk Traditional) sample ((**a**,**d**), respectively). The green inset (**b**,**e**) shows the tooth–restoration interface, which at higher magnification (red inset, (**c**,**f**)) highlights the presence of three different structures: Enamel (E), adhesive layer (Ad) and bulk-fill composite (BFC), without any gap formation on the external margin.

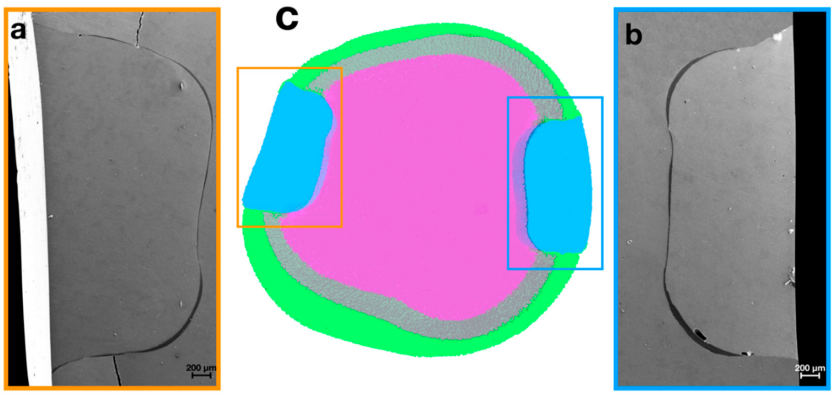

Figure 4. SEM observations of restorations gaps (**a**,**b**) and μ-CT colored 3D reconstruction of a BG representative sample (**c**). Green—enamel; pink—dentin (in transparency the superposition of the dentin with the enamel); blue—restorations.

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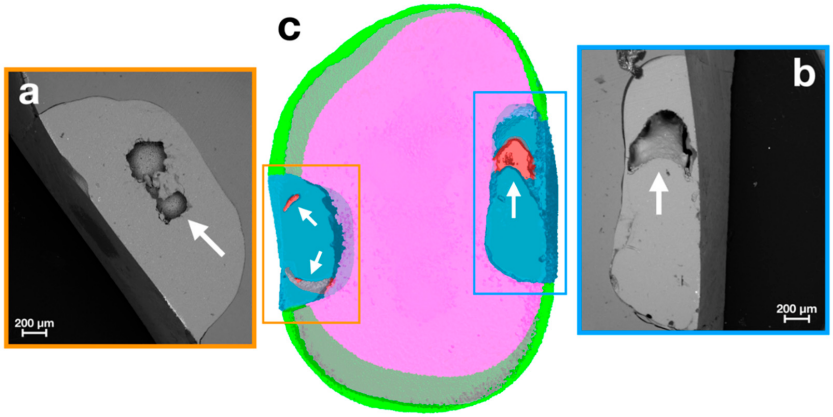
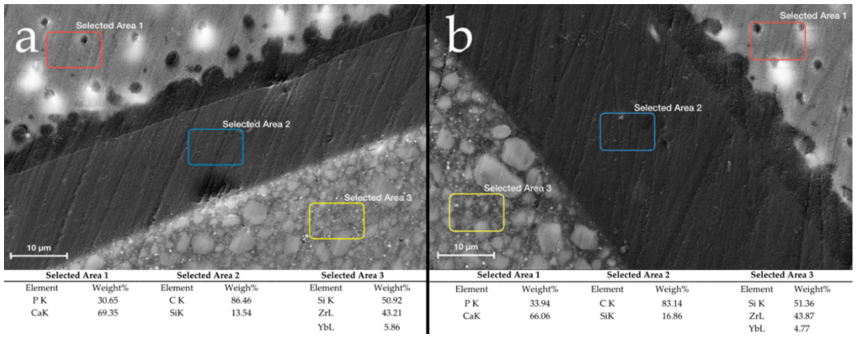


Figure 5. SEM observations of restorations gaps (**a**,**b**) and µ-CT coloured 3D reconstruction of a BT representative sample (**c**). SEM observations of the restorations presenting several defects (arrows in (**a**,**b**)) and µ-CT coloured 3D reconstruction of a BT representative sample with defects (arrows in (**c**)). The void formation in micrograph (**a**) is split in two different defects in the 3D model (orange inset of (**c**)). The void formation in micrograph (**b**) represents the defect inside the restoration (blue inset of (**c**)). Green—enamel; pink—dentin (in transparency the superposition of the dentin with the enamel); blue—restorations (in transparency superposition of the dentin with the restoration); red—defects.

Figure 4 included the 3D model realized by µ-CT (c) and the scanning electron micrographs (a,b) of the internal marginal adaptation of BG restorations. In particular, insets (a) and (b) highlighted the preserved integrity seal, not showing the presence of gaps at the tooth–restoration interface.

On the other hand, Figure 5 showed in both the 3D model (c) and the scanning electron micrographs (a,b), the preserved internal integrity seal with void formation inside of BT restorations.

In all groups, the chemical composition of the tooth–restoration interface was analysed using EDS. Figure 6 reported the levels of the chemical constituents (element and weight in percentage) for the different selected areas, as follows: Selected area 1, corresponding to the enamel surface (E); Selected Area 2, Adhesive layer (Ad); Selected Area 3, BFC surface (BFC).



Selected Area 1		Selected Area 2		Selected Area 3	
Element	Weight%	Element	Weigh%	Element	Weight%
P K	30.65	C K	86.46	Si K	50.92
CaK	69.35	SiK	13.54	ZrL	43.21
				YbL	5.86

Selected Area 1		Selected Area 2		Selected Area 3	
Element	Weight%	Element	Weigh%	Element	Weight%
P K	33.94	C K	83.14	Si K	51.36
CaK	66.06	SiK	16.86	ZrL	43.87
				YbL	4.77

Figure 6. The scanning electron micrographs displayed the three different structure of tooth–interface surface of BG and BT groups ((**a**,**b**) respectively). Selected Area 1: Enamel surface (E); selected Area 2: Adhesive layer (Ad); Selected Area 3: BFC surface (BFC). EDS results of BG and BT groups are reported in the table analysing the different elements as follow: Phosphorous (P), calcium (Ca), carbonium (C), silicon (Si), zirconium (Zr), and ytterbium (Yb).



4. Discussion

Marginal adaptation and microleakage formation represent the most common problems associated with composite materials, thus influencing the longevity of dental restorations [19,27]. Such defects can result from inadequate adhesion at the interface of the restoration, due to the following factors: Polymerization shrinkage, degradation of adhesive components because of insufficient light curing, different thermal expansion coefficients between the dental substrate and the composite, finishing and polishing procedures [28]. Nedeljkovic et al. claimed that margins of the class II are, among the restorations, those with higher incidence of secondary caries formation [29]. Moreover, when restorations are placed below the CEJ, the quality of the marginal integrity is uncertain [30]. Findings from the current research indicate that different filling techniques can affect gap formation on the external interproximal margins of class II restorations [23]. The present study focused on the evaluation of internal and external gap formation using two different filling techniques for restoring class II restorations, by means of BFC. A digital non-destructive full analysis of post curing restoration was executed in three dimensions, by using μ-CT. Indeed, μ-CT can be considered a proper method to evaluate BFC, testing new filling techniques and cavity preparations, in an effort to obtain a more favourable C-factor, avoiding PSS and marginal gap formation. Moreover, due to its 3D imaging capabilities, the μ-CT represents the preferred non-destructive method to quantify microleakage, obtaining accurate results on PSS, in agreement with those obtained by dye penetration [31]. The accuracy of the μ-CT 3D imaging has been also shown to allow the visualization of bubbles, which cannot be rendered by conventional methods, such as replica techniques [32].

Unlike other published studies [23], using optimized settings, it was possible to maximize the contrast between the densities of the different enamel, dentin and composite resin materials, in just a single scan, so that it was not necessary to perform scans before and after restoration, saving experimental time and vanishing possible artefacts introduced by the synchronization process. The Defect Analysis Tool of VG Studio MAX software has been calibrated to detect internal defects with a minimum volume of 5000 μm^3, which means minimum linear dimension of around 20 μm. This threshold was chosen to detect in detail also the smallest possible gap formation; indeed, recent studies observed that a gap of about 60 μm may lead to the development of a wall lesion and hence, predispose postoperative sensitivity and secondary caries [22,23,29,33]. Furthermore, noteworthy is the study of Kuper et al., highlighting that threshold for secondary wall lesion may occur in the range of about 0 to 70 μm [34]. Most of defects were observed inside and in the marginal walls of the restoration, as also reported by previous studies [35–39] and, to a lesser extent, they were found on the mesiodistal and buccolingual walls of restorations. This finding is not completely in agreement with the results of previous studies, which correlated greater contraction in free surfaces (occlusal walls) than in the remaining walls [35,38].

Regarding the number of the total evident defects (Figure 2a), it can be observed that the average value of both BG and BT groups are similar; however, the SD varies greatly, passing from a unit value in the case of BG to 11.5 in the case of BT. This relevant mismatch should be emphasized, as it indicates how the BG technique has more reproducible results regardless of the operator experience and skill.

The characteristics of the observed defects were defined by the following three overall parameters: The specific surface of the defect (DS/DV), the average diameter of the defects (Mean Thickness), and the ratio between the total volume of defects and the absolute volume of the material (DV/MV). For each of them, the post-hoc power analysis clearly showed that the sample size was more than sufficient to support our conclusions. Regarding the morphometry of the defects, by analysing the impact of these parameters individually, it can be observed that the mean thickness (Figure 2c) shows average values strongly in favour of the BG technique, as they are significantly lower and with smaller standard deviations. This presence of smaller gaps in BG samples, combined with the data referred to the total number of defects, leads to a lower total volume of defects. These data are included in the evaluation of the DV/MV parameter, expressed as a percentage



(Figure 2d), and it is still clearly in favour of the BG group, with values up to 10 times lower than the BT group. However, μ-CT revealed the presence of bubbles inside the restorations of the BT group, possibly responsible of the higher DV/MV ratio.

The defect specific surface (DS/DV), defined as the surface area per unit volume, is a morphometric parameter that remarkably contributes to the study of microleakage and bubbles formation during restorations. This measure is strongly dependent from the defect's shape. Interestingly, we found that the defect specific surface (DS/DV) parameter revealed fundamental differences between the two groups. In the BG group, the mean value is higher than in BT group, thus implying a more oriented defect shape (like a layer); conversely, the BT lower values of specific surface indicate the presence of defects with spherical shape, i.e., the bubbles found inside the restorations. Indeed, SEM and μ-CT images confirm these findings, thus displaying, for the BG group, exclusive presence of defects with a laminar shape (Figure 4), while for the BT group, also there is presence of the bubbles with sphere-like-shapes (Figure 5). The laminar shape observed for BG is correlated to defects extended to the cavity wall; while for BT, the spherical shape of the defect inside the restoration is correlated to the possibility of air bubbles entrapment into the restoration. This fact is explained by the different cavity filling techniques, in which the application of different layer (BT) involves certain risks such as the air bubbles incorporation or contamination between layers [40].

A limitation of the μ-CT technique is that materials without sufficient radiopacity and with low or no filler content, as for dental adhesives, are difficult to be visualized through the software reconstruction [41,42]; consequently, discrimination between adhesive and air is challenging by means of this method [43]. Therefore, analyses based solely on μ-CT data are not sufficient to definitely state if defects highlighted on the internal margin are really constituted by gap formations or are the areas filled with the adhesive material. In this context, SEM analysis allowed to evaluate, at higher magnifications than μ-CT, those areas where the μ-CT had indicated a hypothetical gap formation. In fact, from a careful SEM visualization in the axial plane of samples, no real gaps were found at the interface, because most of gaps detected by μ-CT analysis were attributable to adhesive layer with a thickness around 40 μm [44]. Therefore, in both techniques (BG and BT) there were not found relevant formations of defects on the internal margin, but just the adhesive layers. To confirm these visual data, a chemical analysis has been carried out, which has confirmed the presence of the exact chemical elements of the adhesive system used for the restoration (Figure 6).

Therefore, the results of the present research may clearly support the BG technique. Noteworthy is the relationship between the C-factor and microleakage [45]. When the clinician deals with a class II cavity, it would be useful to firstly restore the interproximal wall, in order to transform the class II cavity into a class I, although it has a higher C-factor. Without transforming the class II into class I (contrary to BT technique), a more favourable C-factor is obtained, and hence, a less possibility of deleterious effects on marginal integrity and gap formation. Moreover, the application of the modern formulation of BFC, which can be polymerized up to a depth of 4–5 mm, also showing superior mechanical and optical properties (resulting in better aesthetics) [46–49], allows to reduce the application steps, in comparison with the BT technique, thus minimizing possible clinician's operational errors and chairside time [25].

5. Conclusions

In conclusion, the two bulk-filling techniques, BG and BT, do not show significant differences in the internal and external marginal adaptation of the restoration. The amount and distribution of microleakage visualized in the μ-CT images are closely related to the positioning technique of the composite material. As shown by the μ-CT and SEM analysis, in BT there is the greatest probability of incorporating voids into the restoration.

In general, the standard deviation associated to several morphometric parameters was significantly higher for the BT group than for the BG group, indicating that the



reproducibility of the BT filling technique has a great variability (which means that every time the technique is reproduced, there could be a different result). This variability depends on several factors as the type of material, the type of cavity and the operator. The proposed BG technique has characteristics that make it a valid alternative to the traditional BT technique, like smaller size of defects and lower ratios between total volume of defects and the volume of the tooth.

These results suggest that in deep class II cavities, the Bulk&Go filling technique can represent a reliable alternative to traditional filling technique, hence, without transforming class II in class I. Moreover, the association of the performed evaluations allows to achieve a robust correlation between 3D reconstruction, imaging and chemical data, providing reliable and objective information both on the morphology and the chemical composition of the analysed samples. Regarding the tooth–restoration interface, this approach can be considered particularly useful, giving the opportunity to evaluate the effect of restorative procedures from different point of view, despite further studies are needed to confirm the results obtained.

Author Contributions: Conceptualization, V.T., F.V., and A.P.; methodology, A.P., A.G., and G.O.; software, M.F. and M.L.G.; validation, A.G. and G.O.; formal analysis, M.F. and M.L.G.; investigation, V.T. and F.V.; resources, A.P. and A.G.; data curation, R.M., A.G., and G.O.; writing—original draft preparation, V.T. and F.V.; writing—review and editing, V.T., F.V., R.M., G.O., and A.P.; visualization, A.P.; supervision, A.G. and G.O.; project administration, A.P. and A.G.; funding acquisition, G.O. and A.P. All authors have read and agreed to the published version of the manuscript.

Funding: SISOPD (Società Italiana Stomatologia, Odontoiatria e Protesi Dentaria-Italian Society of Dentistry, Stomatology and Prosthodontics) Fundation partially supported this study. The sponsor had no role on design, analysis, and interpretation of the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author G.O.

Acknowledgments: The authors extend their gratitude to Adriano Di Cristoforo for his precious support in SEM measurements and acknowledge CISMiN facilities of the Polytechnic University of Marche, Ancona, Italy.

Conflicts of Interest: The authors declare no conflict of interest.

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Essential Lines: a simplified filling and modeling technique for direct posterior composite restorations

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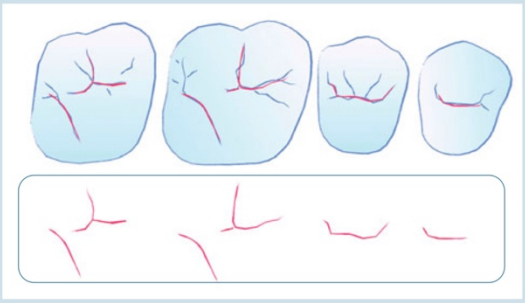
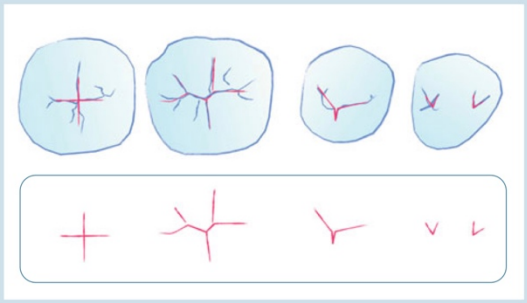
Abstract

There is a continuing increase in demand for the direct restoration of posterior teeth with resin-based composites. However, the execution of these restorations, especially the reproduction of the anatomical details of the tooth, still presents difficulties for most clinicians. Nowadays, resin-based composite restorations have become increasingly popular due to the development of new materials and filling techniques. Direct composite restorations are usually performed using the time-consuming incremental filling technique, which minimizes polymerization shrinkage. With this technique, the composite is built up in individually cured increments. Due to the superior physical and chemical properties of the newer bulk-fill composites, it is possible to replace this incremental filling technique with a more time-efficient bulk-fill technique, whereby the composite is placed and modeled in a single layer up to the occlusal surface. This article presents this novel technique for direct posterior composite restorations. The technique, called Essential Lines, is simple, esthetic, and time saving. It combines the advantages of the bulk-fill technique with a modeling technique that utilizes minimal so-called diagram lines to restore the tooth anatomy. This procedure can be used by beginners and expert practitioners alike in various clinical situations to produce direct posterior composite restorations with optimal function and esthetics.
(Int J Esthet Dent 2021;16:2–5)







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Introduction

Although the restoration of decayed posterior teeth is one of the most common daily dental clinical treatments,[1,2] it still represents a difficult challenge for clinicians. Due to the recent development of new monomers and fillers, resin-based materials have become the gold standard for direct restorations.[3] Moreover, numerous reports have demonstrated that the clinical survival of posterior composite restorations using traditional resin-based composites may be > 90% after 5 years and > 80% after 10 years.[4,5] With the incremental filling technique, direct composite restorations are built up in increments, whereby each layer is cured separately.[6] Several investigations[7,8] have reported that this technique decreases the polymerization shrinkage stress (PSS); however, its drawbacks are that it is time consuming and increases the potential of voids forming between the layers, which is a risk factor for restoration failure.[7-9] To overcome these limitations, bulk-fill composites (BFCs) have recently been introduced. Due to their new monomer content, BFCs can be cured in a maximal increment thickness of 4 mm with limited shrinkage, thus allowing clinicians to fill cavities in one single step with satisfactory cavity adaptation, simplifying the procedure and decreasing the chair time required to perform the restoration.[7,10,11]

Depending on their consistency, BFCs are classified into high and low viscosity. The physical properties of high-viscosity BFCs allow the restoration to be finalized without the further application of a final occlusal capping.[12] On the other hand, low-viscosity (also known as flowable) BFCs easily adapt to any surface irregularities present on the cavity floor, which results in a standardized cavity being obtained, especially in clinical cases where there is less accessibility. The low filler content of low-viscosity BFCs renders the surface less wear resistant. Therefore, a final occlusal capping with a high-viscosity BFC is required with their usage.[13] Conversely, high-viscosity BFCs can be used alone to fill a cavity up to the occlusal surface.

Although the clinical properties and application of BFCs have been widely reported,[11] as far as the present authors are aware, there is no consensus and nothing in the scientific literature describing a technique that provides a simple, fast, and esthetically acceptable modeling procedure based on the advantageous properties of these innovative materials.[11] Therefore, this article presents a novel technique, Essential Lines, which reproduces the occlusal morphology of the posterior teeth when executing direct posterior composite restorations. The technique provides a simple, reproducible, and quick method to produce satisfactory direct posterior composite restorations using BFCs that provide adequate masticatory function and esthetics. The concept behind each step of the Essential Lines technique as well as some clinical case examples are presented in this article.

Technique presentation

'Read' the tooth

It is well known in the profession that an accurate analysis of the occlusal surface anatomy is the crucial first step in direct posterior composite restorations. Before cavity preparation, it is necessary to 'read' and memorize all the anatomical details of what remains of the tooth. Equally important is to review the tooth anatomy when the cavity preparation has been completed but not yet filled, because some of the anatomical features are usually lost during both the cavity preparation and the filling. Indeed, the clinician could conceal the existing morphology after the placement of the entire material at once, as is suggested

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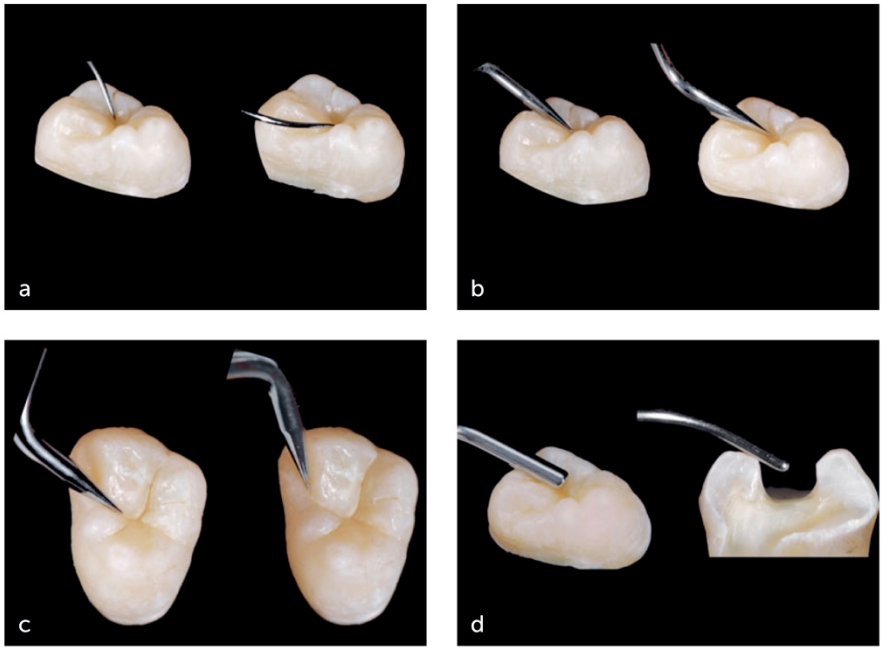

Fig 1 Analyzing the position of the crests, pits, and grooves facilitates the reproduction of the proper occlusal anatomy. The LM-Arte Fissura instrument allows for the detailed exploration of the occlusal morphology. (a) The finest tool tip makes it possible to analyze the depth of the grooves and pits. (b and c) The largest tool tip allows for the study of the triangular, oblique, and marginal ridges. (d) The LM-Arte Condensa instrument allows for the inspection of the orientation and inclination of the crests.

when using BFCs. As is shown in Figure 1, the angle of the cusps and the position of the primary grooves are the most important details to be copied. If the tooth to be restored is completely decayed or has an extensive preexisting restoration, it is recommended to observe and copy the anatomy of the adjacent and/or contralateral teeth.

Cavity configuration

After removing all the decayed hard tissue, it is recommended to perform a standardized cavity configuration to simplify the reconstructive procedure. For instance, when presented with a Class II cavity, in order to correctly reproduce the marginal ridge, it would be useful to copy the shape and height of the adjacent tooth when building up the interproximal wall. This will turn the Class II into a Class I cavity. An innovative instrument, the LM-Arte Posterior Misura (LM-Dental) can be used to achieve this. After placing a matrix, the fork-shaped tip of this instrument measures and models the BFC, copying the shape of the adjacent tooth wall, as shown in Figure 2.

Once the marginal ridge is restored and a Class I cavity is obtained, it is essential to check the cavity and measure its depth to



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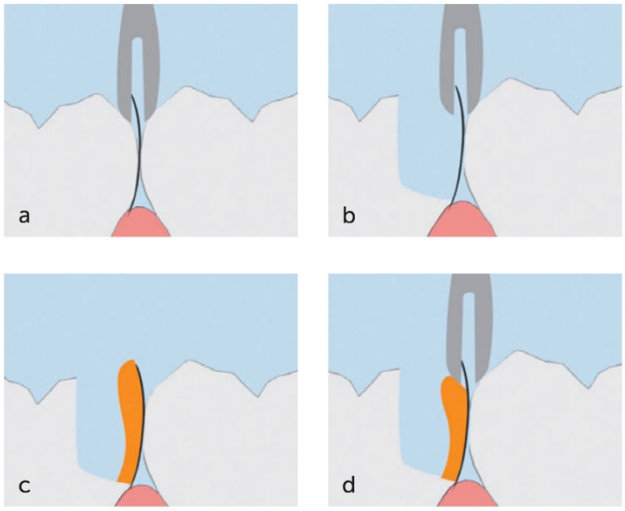

Fig 2a to d Graphic showing the usage of the LM-Arte Posterior Misura to restore the proximal wall of a posterior tooth. After the placement of the matrix band, the forked-shaped tip copies the marginal crest of the adjacent tooth, reproducing its height and the contour of the coronal aspect of the contact point.

choose the best filling technique. If the cavity is deeper than 4 mm, it is advisable to first use a low-viscosity BFC to seal, cover the undercuts, and regularize the cavity floor (Fig 3). After this step, the cavity can be filled up to the occlusal surface using a high-viscosity BFC.

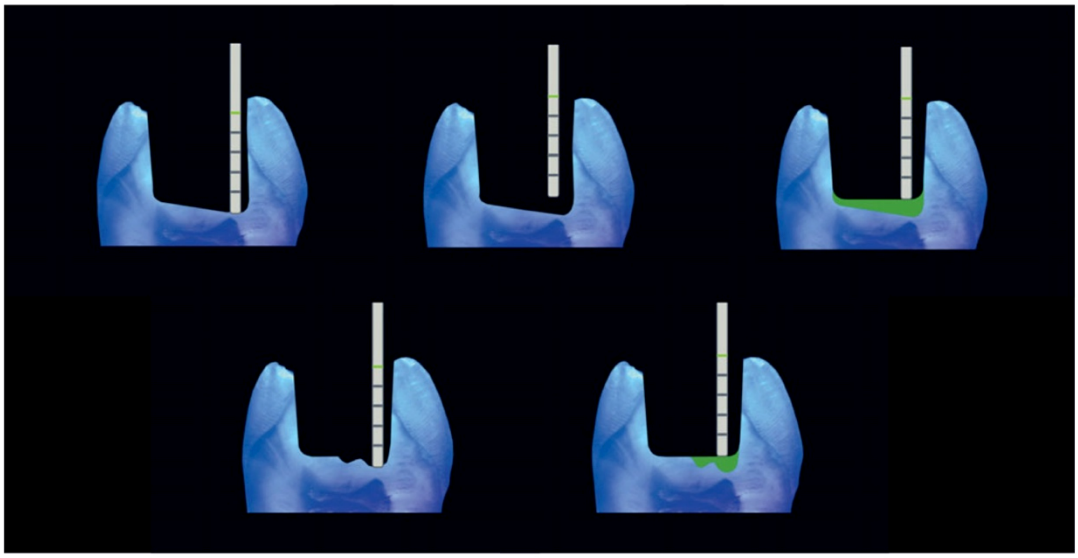

Fig 3 Once the carious lesion is removed, it is necessary to measure the cavity depth and analyze the cavity floor. If a depth of more than 4 mm is measured using a periodontal probe, it is suggested to fill the cavity with a thin layer of low-viscosity BFC, which will regularize the cavity floor and obtain a standard preparation.

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Filling techniques

If the cavity measures approximately 4 mm in depth, there are two different Essential Lines techniques that can be used to fill it, depending on the clinician's preference, the patient's esthetic expectations, and the materials and time available. It is noteworthy that, regardless of the clinical case, it is always advisable to perform the last layer and the occlusal modeling simultaneously.

The fundamental filling procedures of Essential Lines include:

1. The 'Bulk and Body' technique, which is a two-step procedure based on the placement of a first layer of high-viscosity BFC, which should mainly act as a dentin, leaving the appropriate space for a second, capping layer consisting of an enamel-like composite (Fig 4). It can be performed using the LM-Arte Posterior Misura instrument, designed for vertically measuring the necessary 1.5-mm–thick layer that will maintain the space for the final enamel composite layer (see Fig 4). This last occlusal layer should have a greater optical density than a traditional enamel composite. Most BFCs have the limitation of being too translucent, which would result in a gray appearance of the final restoration. Therefore, to improve the esthetic result, it is advisable to use a body shade composite with low translucency and a high value for this final layer.
2. The 'Bulk and Go' technique goes beyond the Bulk and Body concept, allowing a one-step procedure, which takes full advantage of the favorable chemical–physical characteristics of the newly developed high-viscosity BFCs. This second technique thus obtains a satisfactory degree of conversion throughout the entire single layer. Therefore, providing the cavity does not exceed a depth of 4 mm, its restoration can be realized in a single mass application with simultaneous modeling (Fig 5).

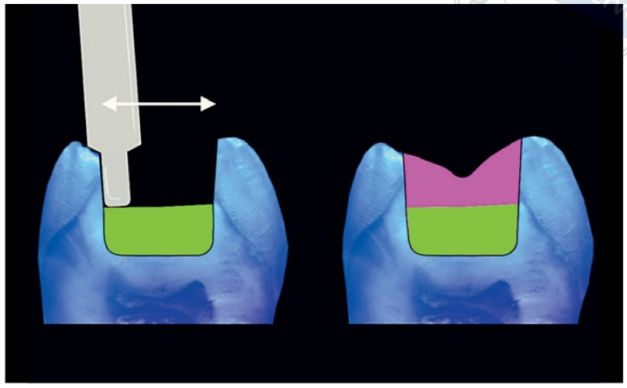

Fig 4 Graphic showing how the LM-Arte Posterior Misura tip works. The instrument allows the cavity to be filled with a first dentin-like BFC layer (green), leaving a 1.5-mm–deep space for the second and final occlusal layer consisting of an enamel-like composite (pink). After placing these two increments, the technique is completed by the final simultaneous modeling of the occlusal surface, which reproduces the tooth anatomy.

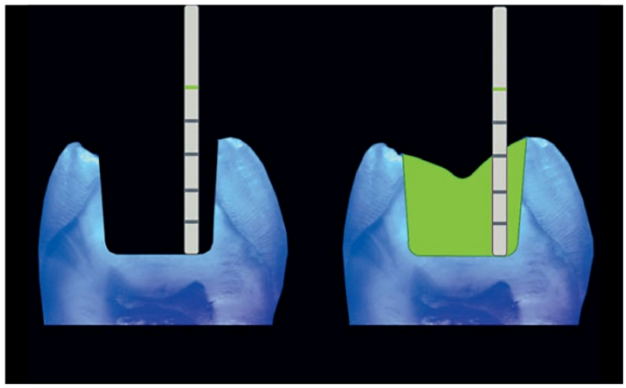

Fig 5 Graphic showing the situation when the cavity has a depth of ≤ 4 mm. In this case, it is possible to fill the cavity completely with a single layer of high-viscosity BFC. This material allows the restoration of the tooth to be performed in only one step, with the simultaneous modeling of the occlusal surface.



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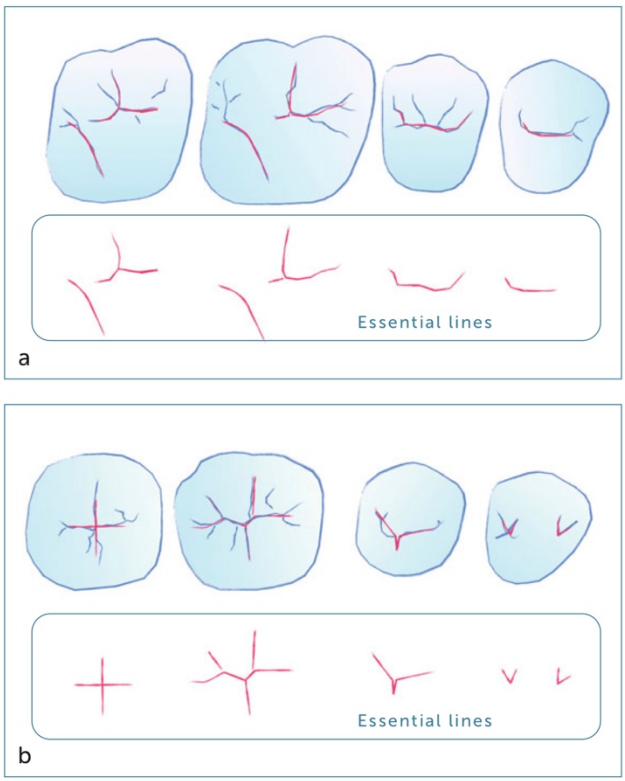

Fig 6 Diagrams showing the drawing of the occlusal Essential Lines of the posterior teeth. (a) The Essential Lines of the maxillary posterior teeth. (b) The Essential Lines of the mandibular posterior teeth. From left to right: second and first molars, and second and first premolars, respectively.

Modeling technique

The essence of the Essential Lines technique consists of drawing minimal lines on the last application of the composite in order to model a tooth-like restoration (Fig 6).

Once the cavity has been filled and the material adapted, it is mandatory to remove the excess, allowing a full display of the residual anatomy. An ideal instrument to perform these steps is the LM-Arte Condensa (LM-Dental). The rounded working end allows the material to be condensed, and the angulated shank allows it to be spread on the margins with a movement from the center to the periphery (Fig 7).

After building the cusps, a plain occlusal surface will be obtained onto which the Essential Lines will be drawn. The first step of modeling will be the identification of the central fossa, which represents the start from which every line should be drawn, always from the center to the periphery of the restoration. The anatomy of the sulcus and the cusps is performed on the composite using a thin pointed instrument, such as the LM-Arte Fissura (LM-Dental), which highlights the anatomical details of the cusps (Fig 8). It is important to avoid dragging the material when drawing the lines, otherwise it is not possible to maintain the correct shape. The lines should be drawn as a series of several point in order to press the material without stretching it, following the movement known as step-walking (see Fig 8).

Tips for modeling

After finishing the design of the occlusal anatomy, it is possible to open or further accentuate the grooves to avoid the final restoration having a flat occlusal surface. The grooves can be opened or closed by touching the material with the LM-Arte Fissura instrument at the level of the groove, as shown in Figure 9.

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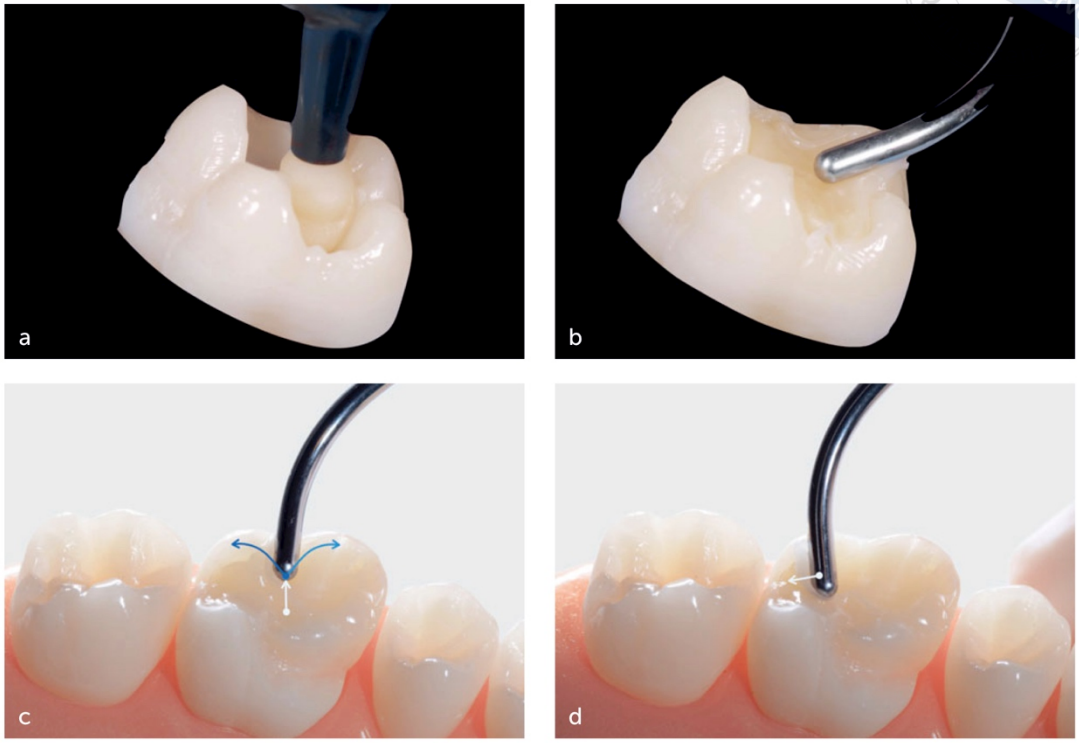

Fig 7 a to d Images showing the correct movement of the LM-Arte Condensa tip. Once the cavity is filled, it is recommended to adapt the composite by removing the excess with the large tip of the instrument. Note: the instrument should move from the center to the periphery (arrows).

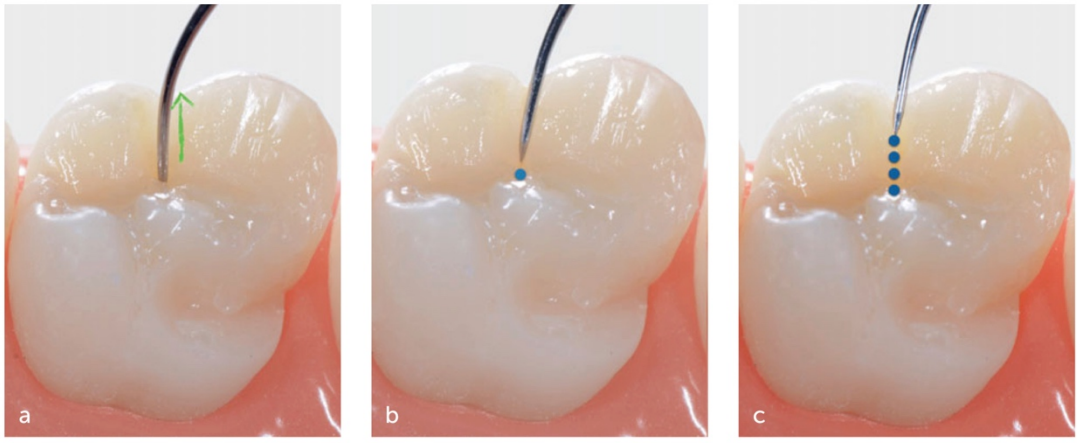

Fig 8 a to c Images showing the correct movement of the LM-Arte Fissura tip. Once the composite has been adapted, it is important to draw the sulcus as a series of points to form a line, using the finest tip of the instrument.

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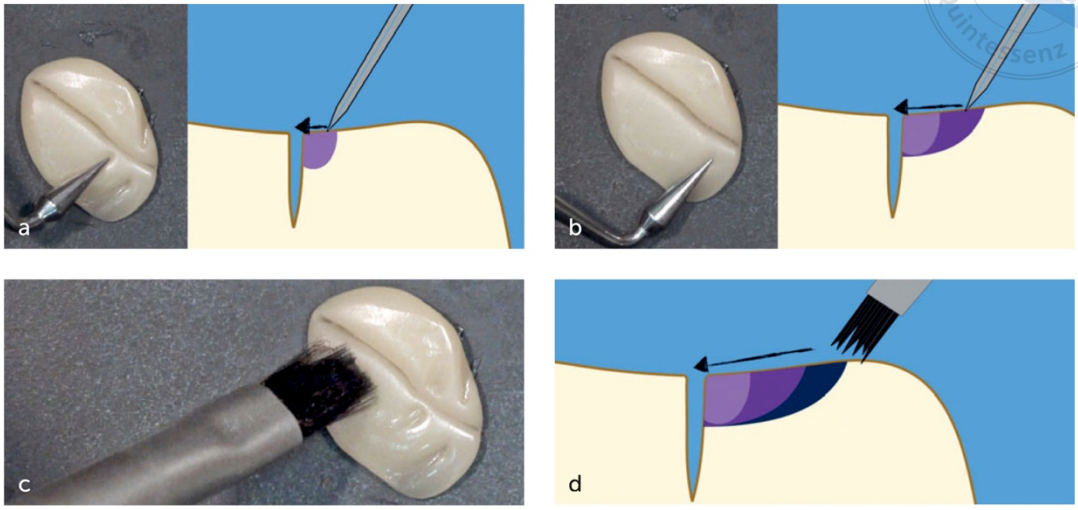

Fig 9a to d Images and graphics showing how the grooves can be opened or closed. The closer the instrument is to the groove, the less material can be moved, and the further it is from the groove, the more material can be moved. If the groove becomes too closed, it can be reopened by retracing the lines. A brush can also be used to gently move the material and make the anatomy smoother. If the desired result is not achieved, it is possible to erase the grooves with a small brush or with the LM-Arte Condensa and begin again.

The closer the instrument is to the groove, the less material it moves; conversely, the further it is from the groove, the more material it moves. If the grooves become too closed, they can be reopened by retracing the lines using the LM-Arte Fissura instrument. It is possible to move much more material by using a brush instead; moreover, brushes and micro brushes can also be used to soften the obtained anatomy. If the desired result is not achieved, it is possible to erase the groove design using a small brush or the LM-Arte Condensa instrument and begin again by redrawing the grooves, always starting from the center.

Clinical cases

The five clinical cases presented in Figures 10 to 20 provide step-by-step clarification of the Essential Lines technique. It is possible to choose either the two-step (Bulk and Body) or the one-step (Bulk and Go) procedure and to finally draw the lines onto the occlusal surface. The Bulk and Body technique is described in Cases 1 and 2, and the Bulk and Go technique in Cases 3 to 5. All the cases were carried out after careful radiographic evaluation and the patients' signed informed consent.

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Case 1



Fig 10 (a) Preoperative bitewing radiograph of tooth 16. (b) Occlusal view of the occluso-mesial amalgam restoration. The patient had requested its replacement for esthetic reasons. (c) The old restoration is removed and the selected bonding system applied. The tooth is then reconstructed following the Bulk and Body technique. (d) After transforming the Class II into a Class I cavity using a metal matrix (white asterisk), a flowable BFC is placed to obtain a flat floor cavity.

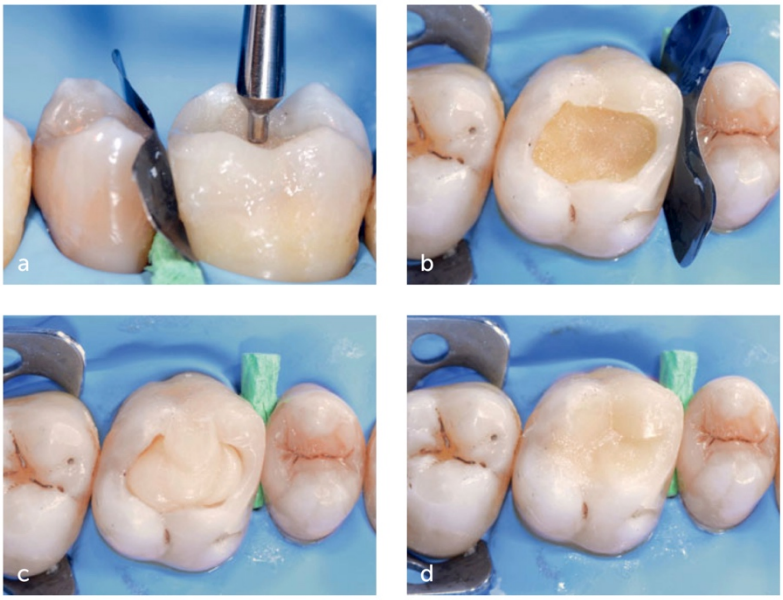

Fig 11 (a) A single apposition of a high-viscosity BFC is performed using the LM-Arte Posterior Misura instrument, leaving a 1.5-mm–thick space for the final layer. (b) Occlusal view of the BFC single-mass layer placement. (c) A final occlusal body composite layer is placed. (d) Adaptation of the body layer is performed by first removing the excess and spreading the material toward the margin of the restoration.

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Fig 12 (a) After the composite adaptation, the Essential Lines of tooth 16 are drawn. It is crucial to plot the lines as a series of several points to avoid dragging the composite. (b) Once the drawing of the lines is complete, stains are performed to ensure a more 3D appearance of the restorations. (c) Postoperative final result after rubber dam removal. (d) Restoration at the 1-year follow-up.

Case 2

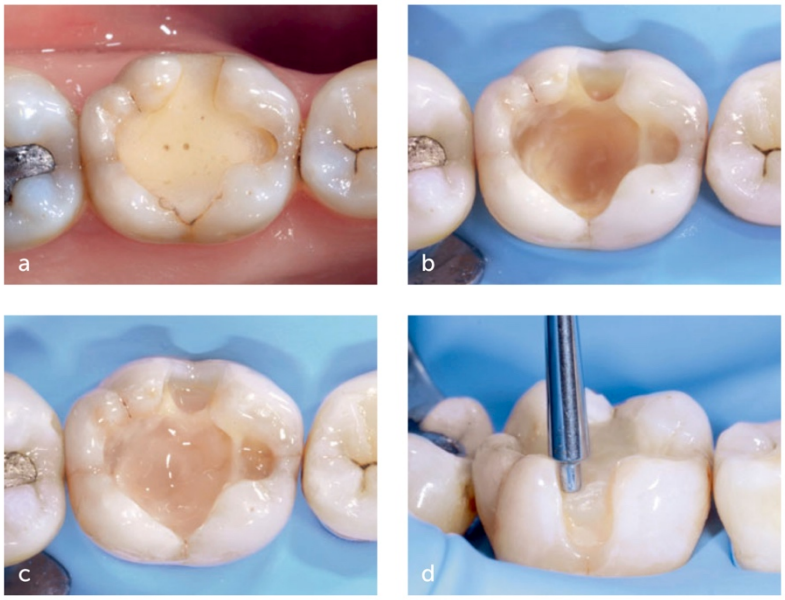

Fig 13 (a) Preoperative occlusal view of the defective restoration of tooth 36. (b) Rubber dam application and removal of the previous composite and the decayed tissue. (c) After selective enamel etching and the application of universal bonding agent, a flowable BFC is placed to obtain a flat floor cavity. This standardized cavity it is now ready to be filled. (d) The Bulk and Body procedure starts with a single application of a high-viscosity BFC. Once the BFC has been compacted and adapted, the I.M-Arte Misura Posterior instrument is used in order to leave the 1.5 mm required for the final capping layer.

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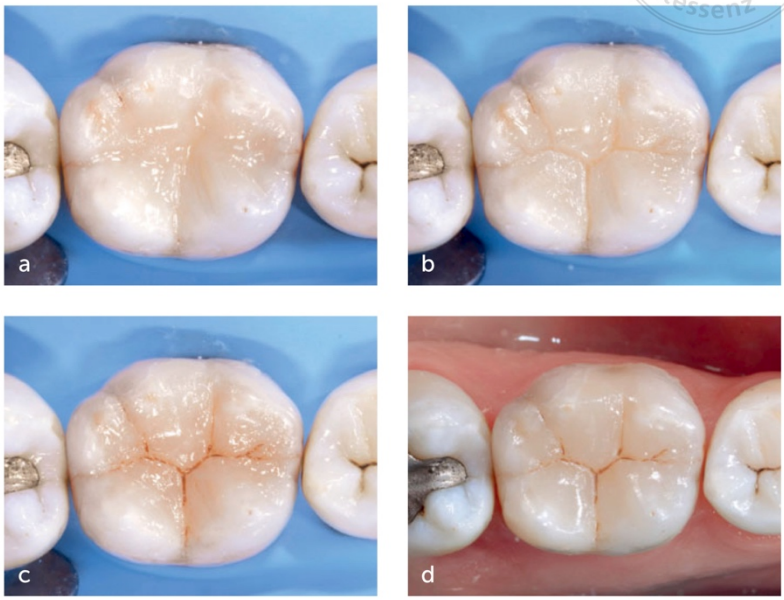

Fig 14 (a) Placement of the capping layer made of an enamel-like material body composite. (b) The Essential Lines are drawn on tooth 36. (c) It is then possible to perform stain characterization depending on esthetic demands. (d) Postoperative view of the final restoration.

Case 3

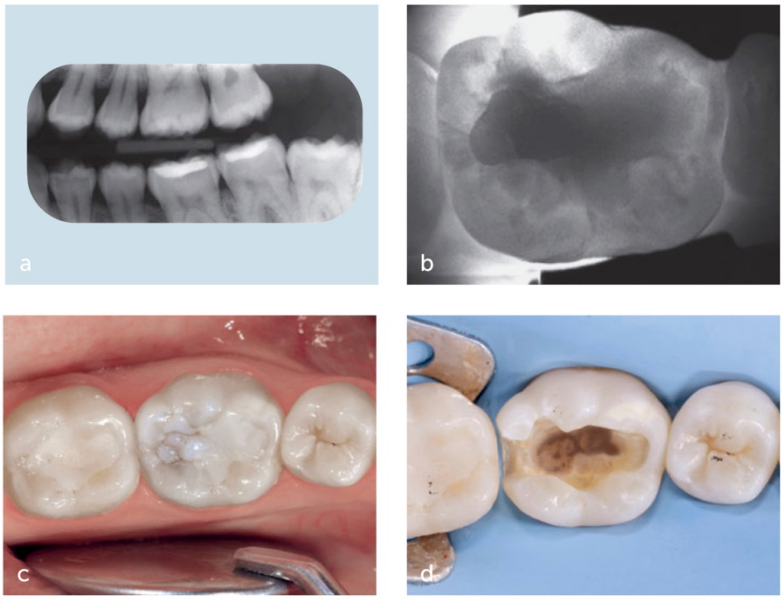

Fig 15 (a) Preoperative radiograph showing the preexisting restorations on teeth 36 and 37. (b) The near-infrared light transillumination by DIAGNOcam (KaVo Dental) shows a carious lesion on tooth 36 localized under the composite restoration, also including the distal wall. (c) Preoperative occlusal view of tooth 36 affected by secondary caries, and the poorly carved restoration of tooth 37. (d) After rubber dam placement, the old composite restoration of tooth 36 is removed, producing a Class II occluso-distal cavity.

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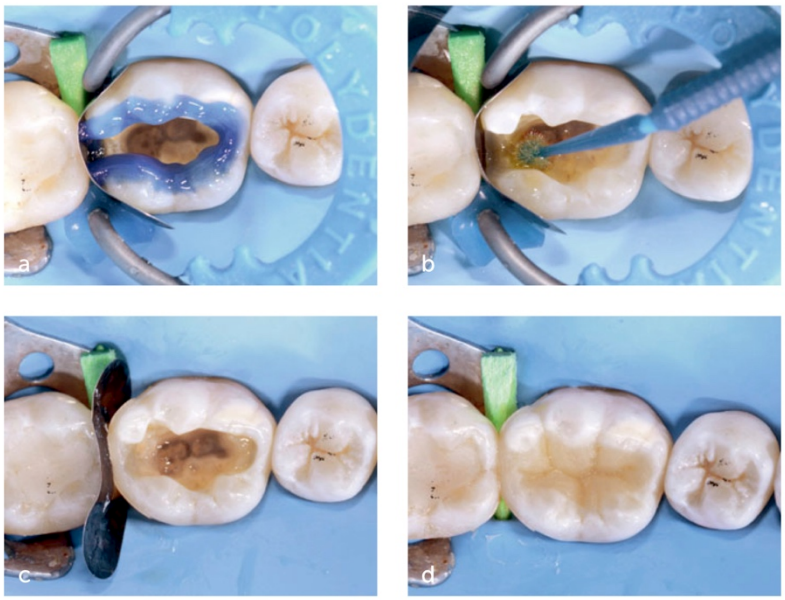

Fig 16 (a) Selective enamel etching using 37% orthophosphoric acid. (b) Application of a universal bonding agent to both the enamel and dentin. (c) Restoration of the distal proximal wall of tooth 36 using a high-viscosity BFC. After checking the depth of the cavity, the Bulk and Go technique is used for tooth reconstruction. (d) The cavity is filled up to the occlusal surface with the same BFC used for the proximal wall. After setting the position of the central fossa, the Essential Lines are drawn onto the occlusal surface. Setting the lines using the step-walking movement (described in the text of the article) is crucial to avoid the material dragging.



Fig 17 (a) After the restoration of tooth 36 is completed, a Class I cavity is prepared on tooth 37. (b) The Bulk and Go technique is also performed in this case. The cavity is filled up to the occlusal surface. (c) After marking the central fossa, the Essential Lines are drawn on tooth 37. (d) Postoperative view of the final restorations.

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Case 4

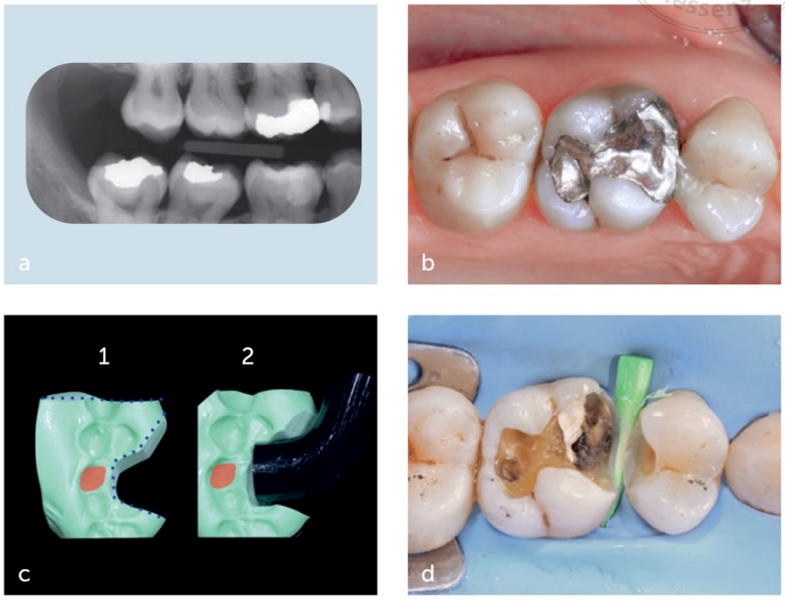

Fig 18 (a) Preoperative radiograph showing a distal lesion on tooth 15 and a mesial lesion on tooth 16. (b) Preoperative occlusal view of the suboptimal contact points between teeth 15 and 16. (c) A silicone index is taken to record the proper height and position of the tooth cusps. The silicone index is then cut in order to leave the space for the curing lamp tip [1]. The orange area represents the space for the composite placement, while the blue dotted line shows where the silicone index guide has been cut. The curing lamp tip fits into the prepared silicone index, allowing a closer contact between the tip and the tooth [2]. (d) Once Class II cavities have been prepared, the rubber dam and wedge are placed.



Fig 19 (a) The distal wall of tooth 15 is restored using a high-viscosity BFC. The height of the mesiobuccal cusp of tooth 16 has been reduced, since the enamel was not supported by dentin. (b) The mesiobuccal cusp is restored using the silicone index. The curing tip is placed within the prepared silicone index. (c) The high-viscosity BFC is placed to restore the mesial wall to transform the Class II into a Class I cavity. A ring with shaped silicone ferrules and a sectional matrix has been used. (d) Measurement of the cavity depth; as it is 4 mm, the cavity can be filled using one single BFC layer.

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Fig 20 (a) After placing the BFC, the excess material is removed from the margins. (b) Once the central fossa is identified, the Essential Lines of the maxillary first molar are drawn. (c) Postoperative final restoration, immediately after rubber dam removal. Brown staining of the sulci can be made to improve the esthetic result. (d) Occlusal view of the restored teeth at the 3-month follow-up for the restorations on teeth 15 and 16.

Discussion

The Essential Lines technique proposes a simplified filling and modeling technique that takes advantage of the benefits of BFC.[14] Recent studies have shown that the new BFCs perform similarly or better than traditional composites.[15-17] Indeed, both high- and low-viscosity BFCs can overcome the limitations of the incremental layering technique without compromising the PSS, mechanical properties, and degree of conversion.[1,18-22] El-Damanhoury and Platt[7] reported a significant reduction in PSS while maintaining comparable curing efficiency at 4 mm for some BFCs, thus supporting the potential use of BFCs for posterior restorations. Furthermore, no significant differences in the marginal gap formation and marginal integrity failures were observed when BFCs were compared with traditional materials.[23] Ersen et al[24] reported an acceptable shrinkage range of BFCs, with filling and physical properties comparable to conventional composites. Rosatto et al[10] reported that the use of BFCs in posterior restorations reduced cusp deformation, post-gel shrinkage, and shrinkage stress, and increased the fracture resistance. Therefore, BFCs can be used in both high or low deep cavities. Noteworthy is that although the description of the Essential Lines modeling technique is based mainly on BFC use, it can also be performed using conventional materials, especially when dealing with 2- to 3-mm–deep cavities.

In the Essential Lines filling technique, low-viscosity BFCs are used to both regularize the floor of the cavity and decrease its depth. Several studies have shown that flowable BFCs have better marginal adaptation and less volumetric polymerization shrinkage than traditional composites.[21,25-29]



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In a meta-analysis by Cidreira Boaro et al,[30] flowable BFCs showed lower shrinkage than conventional ones.

In the technique presented in this article, flowable BFC is used only on the floor of the cavity, whereas high-viscosity BFCs are preferred over low-viscosity ones from a practical point of view, since they easily allow for the modeling of the composite and the removal of excess in case of overfilling. On the other hand, in case of a flowable BFC, the removal of excess would be very difficult and time consuming. In addition, it would be difficult to measure the cavity height.

In order to further simplify and accelerate the filling procedure, while the Bulk and Body technique requires a superficial body composite layer over the BFC, with the Bulk and Go the entire cavity is filled using a single BFC. The main reason to propose the Bulk and Body technique is the esthetic requirement of the patient. Indeed, the single placement of some BFCs, although fast and simple, may lead to a poor esthetic that does not meet the patient's expectations. In such cases, a final layer of a body resin composite could improve the final esthetic result.

In summary, in the Essential Lines technique, after completing the cavity preparation, the clinician can use a low-viscosity BFC to obtain a regular cavity floor. Next, according to clinician preference, patient expectations, and available chair time, the Bulk and Body or the Bulk and Go technique can be chosen to fill the cavity. This should preferably, but not exclusively, be performed by means of a high-viscosity BFC. The drawing of the lines is then performed to reproduce the occlusal anatomy of each posterior tooth.

Conclusion

Nowadays, patient demand for high-quality esthetic restorations is high. This can prove to be challenging when restoring posterior teeth. Essential Lines is a simple, predictable, time-efficient technique that all clinicians, regardless of expertise and experience, can follow to produce highly esthetic, anatomical, and functional posterior direct restorations.

Acknowledgment

The authors thank Dr Andrell Hosein for her valuable contribution in the drafting and writing of this article.

Disclaimer

The authors declare that there are no conflicts of interest or relevant financial relationships relating to this article.



CLINICAL RESEARCH

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Minerva Stomatologica 2020 mese;69(0):000-000
DOI: 10.23736/S0026-4970.19.04310-3

ORIGINAL ARTICLE

Effect of four different finishing and polishing systems on resin composites: roughness surface and gloss retention evaluations

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ABSTRACT

BACKGROUND: Finishing and polishing procedures play a crucial role to achieve the best aesthetic result of direct restorations. Many manufacturers to the clinicians offer different finishing and polishing systems. This study aims to examine the behavior of four different finishing and polishing systems on the roughness, gloss and morphology surface of one resin composite.

METHODS: Twelve Filtek XTE Supreme (3M ESPE) discs were prepared and divided into 4 groups (N.=3), depending on the different finishing and polishing systems: Group 1 (GP1) Sof-Lex Extra-Thin XT discs; Group 2 (GP2) Sof-Lex Coarse black disc, multi-fluted bur, Sof-Lex Diamond Polishing System Spirals; Group 3 (GP3) Sof-Lex Coarse black disc, multi-fluted bur, Sof-Lex Diamond Polishing System Spirals, Diamond Twist SCL; Group 4 (GP4) with Sof-Lex Coarse black disc, multi-fluted bur and Occlubrush. The roughness and gloss were evaluated and then samples examined by Scanning Electron Microscopy (SEM) to analyse the morphology after finishing and polishing.

RESULTS: Regarding the surface roughness, GP1 and GP2 showed similar values for Ra, Ry, Rz and Rq parameters; while GP3 displayed the lowest values in all parameters; GP4 had the highest Ra value. Regarding the gloss, the values decreased as follows: GP3>GP2=GP4>GP1 ($P<0.05$). Scanning electron micrographs showed the abrasion of the samples with an increase in the surface roughness in GP1 and GP4. One-way ANOVA followed by Tukey's Test was used for statistical evaluations ($P<0.05$).

CONCLUSIONS: The roughness and gloss surface of resin composites depend on the finishing and polishing systems used. This study demonstrates that satisfactory gloss outcomes are obtained using a system based on diamond paste.

(*Cite this article as:* Tosco V, Monterubbianesi R, Orilisi G, Procaccini M, Grandini S, Putignano A, *et al.* Effect of four different finishing and polishing systems on resin composites: roughness surface and gloss retention evaluations. Minerva Stomatol 2020;69:000-000. DOI: 10.23736/S0026-4970.19.04310-3)

KEY WORDS: Composite resins; Nanocomposites; Dental polishing.

Resin composites have been improved in the last years in terms of physical properties and aesthetic characteristics.[1, 2] In order to preserve the physiological features of the tooth to be restored, it is critical to consider the surface characteristics of restorative materials and trying to reach the same surface roughness, gloss, and color stability of the natural tooth.[3] Nowadays, nanohybrid and nanofilled resin composites are considered universal resin composite suitable for the restoration of anterior and posterior teeth due to their excellent aesthetic properties.[4] It is known that the type and size of the filler particles influence the physical and mechanical properties of the material and protect the organic matrix against the force applied to the direct restoration,



having a direct influence on the surface properties of the composite such as the roughness and surface gloss.[5] For example, in the nanocomposites, the particles are less susceptible to detaching after finishing and polishing procedures.[6] Most resin composite restorations require finishing after placement in order to remove overhangs and excess of resin composite as well as restoring the occlusion and morphology.

Finishing of a restoration is defined as the gross reduction of excess material to achieve the ideal morphology and optimal function. It is achieved by using either fine and extra-fine diamond burs, or multifluted tungsten carbide burs.[7-9] The finishing process, however, increases the surface roughness above the acceptable threshold; polishing is therefore needed to reduce the surface roughness to acceptable values.[4] Polishing of a resin composite restoration is defined as the reduction of roughness and scratches which were created whilst finishing the composite.[9] Due to the importance of the aesthetic results, it would be useful understanding better the finishing and polishing procedures and evaluate their effect on the resin composites, in particular the surface roughness and gloss retention.

The surface roughness and gloss retention represent the most important properties affecting the appearance and the clinical success of dental restorations. Surface roughness has an influence on the amount of plaque accumulation on composite surfaces and plays an important role in the aesthetics of the restoration.[7] Although the patients can detect a surface roughness greater than 0.50 μm with their tongues, a surface roughness threshold of below 0.2 μm is necessary to prevent the risk of biofilm adhesion and maturation, linked with gingival inflammation, surface staining.[6, 10] In literature, the smoothest surface is obtained when resin composite polymerizes against a Mylar strip without subsequent finishing or polishing.[11] However, such surface gives an uneven geometrical light distribution reflected by the surface.

The gloss of resin composite is influenced by the surface roughness: the light reflected off every angle of the surface as well as the particle size of the material, being associated with the amount of light reflected by the material itself.[4] Therefore, a smoother surface has a higher gloss, indicating superior clinical durability and ideal aesthetic appearance, thus inducing optimal optical compatibility between resin composite and the natural tooth enamel.[12]

Due to the importance of such treatments, clinicians can choose a wide variety of finishing and polishing instruments. However, there are different finishing and polishing systems available in the market.[13] Several studies have demonstrated that multi-step aluminum oxide discs produced the best surface smoothness.[14, 15] However, because of the shape of such discs, they provoke a flat surface, and especially in posterior restorations, do not allow to create the right anatomy. In addition, other finishing and polishing systems exist to treat posterior and anterior areas by means of multi-fluted burs, abrasives and polishing pastes used in one, two or multi-steps. In the last years many attempts focused on optimizing the finishing and polishing instruments.[16]

Several *in vitro* investigations were carried out in order to evaluate the effect of different finishing and polishing procedures, to identify the technique that produces the smoothest surface possible and to identify which procedure increases the gloss values and allows to obtain an acceptable morphology of nanocomposites.[17-19] However, no consensus on this topic has been reached yet. Therefore, our *in vitro* study aims to investigate the effect of four different finishing and polishing systems on the surface roughness, gloss and surface morphology on a recent nanofeatured resin composite.

Materials and methods

Specimen preparation

A nanofilled resin composite, Filtek Supreme XTE (3M ESPE, St. Paul, MN, USA; Table I) was investigated. Twelve specimens were prepared (12 mm in diameter and 4 mm depth) and then randomly divided into four groups (N.=3). Each material was injected into a SmileLine composite mold and pressed between two glass slides covered with a transparent Mylar strips to extrude the excess, thus producing a flat surface and excluding the oxygen inhibition during curing. The samples were polymerized for 40 s from



TABLE I.—*Description of the resin nanocomposite used in the study, according to the manufacturer's data.*

Material (Manufacturer)	Type	Matrix	Average particle size	Filler type	Filler loading vol (%)/wt (%)	Shade
Filtek Supreme XTE (3M ESPE, St. Paul, MN, USA)	Nanofilled composite	Bis-GMA, TEGDMA, UDMA, bisphenol A Polyethylene glycol diether dimethacrylate	0.6-1.4 μm 5-75 nm	zirconia/silica nanocluster Silica-Nanofillers (SiO_2)	59.5/78.5	A2B

TABLE II.—*Description of the different Groups (GP), made according to the polishing system protocols used.*

	Finishing and polishing systems	
	Protocol	Material
GP1	Sof-Lex XT (3M, ESPE, St. Paul, MN, USA) Discs	Aluminum oxide flexible discs
GP2	Sof-Lex Coarse Disc	Aluminum oxide flexible discs
	Multi-fluted bur (Komet Dental, Brasseler GmbH and Co., Lemgo, Germany)	Flame Matches 862 diamond series 30 blades, ultrafine
	Sof-Lex Diamond Polishing System Spiral beige and pink (3M, ESPE, St. Paul, MN, USA)	Elastomer wheels impregnated with aluminum oxide
GP3	Sof-Lex Coarse Disc	Aluminum oxide flexible discs
	Multi-fluted bur	Flame Matches 862 diamond series 30 blades, ultrafine
	Sof-Lex Diamond Polishing System Spiral + Diamond Twist SCL (Premier Dental, Plymouth Meeting, PA, USA)	Elastomer wheels impregnated with aluminum oxide Diamond paste
GP4	Sof-Lex Coarse Disc	Aluminum oxide flexible discs
	Multi-fluted bur	Flame Matches 862 diamond series 30 blades, ultrafine
	Occlubrush (KerrHawe, Boggio, Switzerland)	Silicon carbide 5 μm bristle cup 10.000 water-cooling

each surface using a halogen light-curing unit (Elipar Trilight, 3M-ESPE, Seefeld, Germany), according to the manufacturer's instructions, in standard mode at 850 mW/cm^2 irradiance. All specimens were stored in dry conditions at 23 °C for 24 hours before testing. After curing, each sample was mounted on a glass slide and fixed in place with cyanoacrylate glue, then was subjected to 600 grit sandpaper polishing under water cooling for 30 sec in standard protocol. The samples were randomly divided into four groups, for each different polishing technique, as listed in Table II.

Group 1 (GP1) was treated with all grit sequences of Sof-Lex Extra-Thin XT discs (3M, ESPE, St. Paul, MN, USA); group 2 (GP2) with Sof-Lex Coarse black disc, multifluted bur (Komet Dental, Brasseler GmbH and Co., Lemgo, Germany), Sof-Lex Diamond Polishing System Spiral beige and Spiral pink (3M, ESPE, St. Paul, MN, USA); group 3 (GP3) performed with Sof-Lex Coarse black disc, multi-fluted bur, Sof-Lex Diamond Polishing System Spiral beige and Spiral pink, Diamond Twist SCL (Premier Dental, Plymouth Meeting, PA, USA); group 4 (GP4) with Sof-Lex Coarse black disc, multi-fluted bur and Occlubrush (KerrHawe, Boggio, Switzerland). Finally, the polished samples were rinsed, ultrasonically cleaned for five minutes and air-dried, to remove debris on the surface before roughness and gloss evaluation. All specimen preparations, finishing and polishing procedures were performed by the same operator to get standard results. During the polishing, a planar motion, that is a rotational movement with rotation axis of the abrasive device perpendicular to the surface, was adopted to produce the lowest roughness. Each polishing procedure was performed using a low-speed hand piece at 15.000 rpm with constant and repetitive movements to avoid an excessive heat and formation of surface grooves.

Surface roughness measurements

The surface roughness of the samples was determined by a profilometer (Mitutoyo Surftest SJ 201P, Department of Medical Biotechnology, University of Siena, Siena, Italy). Four rough-

ness parameters were evaluated: Ra (the mean line throughout the sampling length), Ry (the maximum depth roughness in 5 sample lengths), Rz (the mean values of maximum roughness of 5 highest picks and 5 deepest valleys) and Rq (the root mean square deviation of the profile evaluated). For each sample, four measurements were performed, rotating the sample 45 degrees.

Gloss measurement

Gloss was determined by a Novo-Curve Glossmeter (Rhopoint instruments, Department of Medical Biotechnology, University of Siena, Italy), calibrated with a reference value of 95 GU. Each sample was fixed into a silicon mold to avoid light interferences and was placed in the centre of the glossmeter. For each sample, four measurements were evaluated at 60 degrees of light incidence and reflection angles, compared to the vertical axis, turning each time the sample by 45 degrees.

Scanning electron microscope observation

All specimens were also examined by SEM (Scanning Electron Microscope Zeiss Supra 40, Department of SIMAU, Polytechnic University of Marche, Ancona, Italy), after polishing procedures. SEM was operated at 30kV and at a working distance of 12 mm. Five pictures for each sample at different magnification were performed (50x, 250x, 500x, 1000x).

Statistical analysis

The significance of the differences between the experimental groups was determine employing a factorial analysis of variance (one-way ANOVA), followed by Tukey's multiple comparisons test. Significance was set at $P<0.05$. The statistical software GraphPad (Prism 8) was used.

Results

The surface roughness and gloss values of each sample showed some differences, according to the applied polishing and finishing systems. Regarding the surface roughness, the following consideration can be drown: 1) GP1 and GP2 showed similar and not statistically different values for Ra, Ry, Rz and Rq parameters ($P>0.05$); 2) GP3 showed the lowest Ra (0.09±0.04 µm; Figure 1A), Ry (0.74±0.29 µm; Figure 1B), Rz (0.17±0.10 µm; Figure 1C), and Rq (0.69±0.21 µm; Figure 1D) values ($P<0.05$); 3) GP4 displayed the highest Ra value (0.28±0.05 µm; Figure 1A) ($P<0.05$), while Ry (1.49±0.36 µm; Figure 1B), Rz (0.31±0.10 µm; Figure 1C), and Rq (1.39±0.26 µm; Figure 1D) values were similar to the ones of GP1 and GP2 ($P>0.05$).

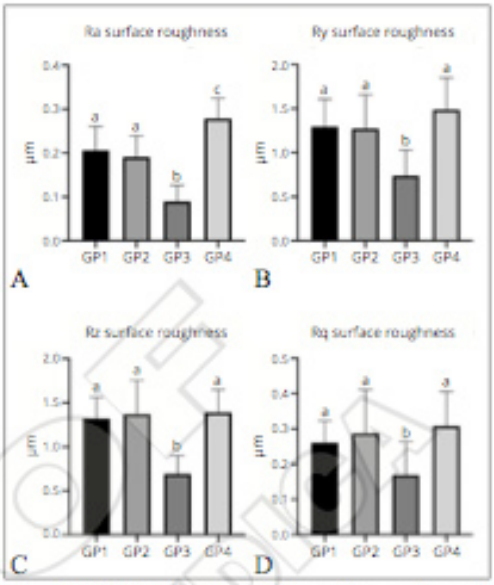

Figure 1.—Statistical analysis of the surface roughness of GP1, GP2, GP3 and GP4 samples: A) Ra; B) Ry; C) Rz; and D) Rq values. Data are presented as mean±S.D. Different letters indicate statistically significant differences among groups.

Regarding the gloss values, the following results were obtained (Figure 2): 1) GP1 showed the lowest values (14.25±4.26 GU) ($P<0.05$); 2) there was not statistical difference between GP2 and GP4 (respectively 26.08±8.60 GU and 22.69±9.63 GU) ($P>0.05$); 3) GP3 showed the highest values (64.98±11.17 GU) ($P<0.05$).

SEM analysis of the samples were also performed (Figure 3). At low magnification (50x) all groups, except GP1, displayed a quite smooth surface. At highest magnification (250x and 1000x), SEM images showed the abrasion and the gloss loss of the samples with an increase of the surface roughness in GP1 and GP4. The

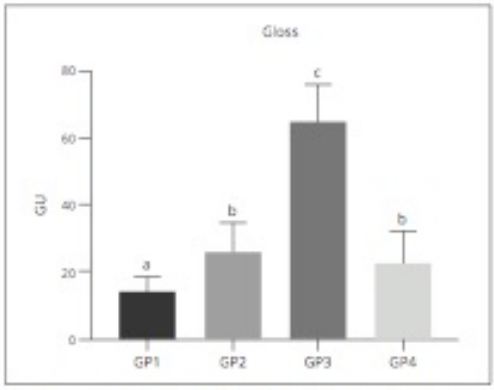

Figure 2.—Statistical analysis of the gloss values calculated for GP1, GP2, GP3 and GP4 samples. Data are presented as mean±S.D. Different letters indicate statistically significant differences among groups.

morphology of GP1 surface seems to be the most irregular, followed by GP4; while GP3 appears to have the smoothest surface, followed by GP2.

Discussion

In this study, the surface roughness, the gloss and the morphological analysis were analyzed after different finishing and polishing procedures.

Smooth surface enables clinical durability, good aesthetic appearance, perfectly tooth-restoration matching and prevents the discoloration and staining of the restoration. The surface roughness and the gloss value, as reported in previous study, depend not only on the microstructure of the resin composite, but also on the type of finishing and polishing techniques used.[20]

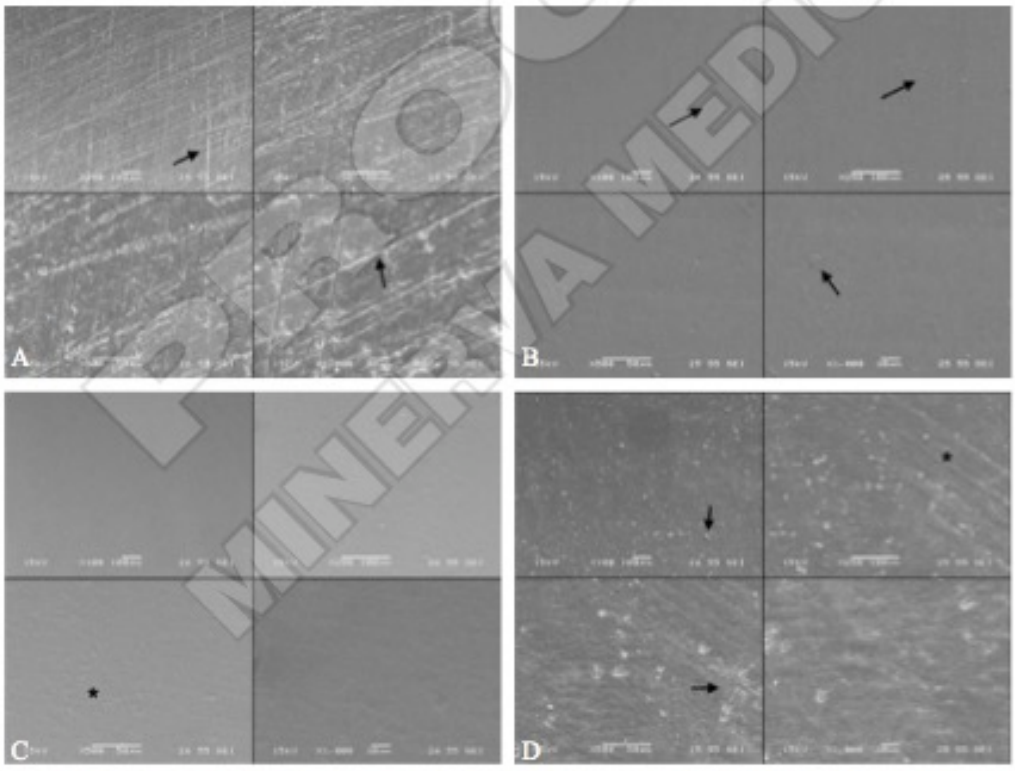

Figure 3.—Scanning electron micrographs, obtained after polishing procedures, in the four groups; (A) GP1, (B) GP2, (C) GP3, (D) GP4. The number after the uppercase letter represents the image magnification:[1] 100x,[2] 250x,[3] 500x,[4] 1000x. Micrographs A show surface irregularities and scratches (arrows). Micrographs B present a regular and smooth surface with the presence of polishing residues (arrows). Micrographs C show a smooth and regular surface with the presence of little depression area (*). Micrographs D present an irregular surface, with the presence of amorphous deposit and depressions (arrows) and residual scratches (*).



Clinical studies have shown that the surface roughness (Ra) of a direct restoration affects the gingival health of the restored tooth, due to both poor oral hygiene and increased plaque accumulation.[18] However, the surface roughness can only be discriminated by patients when the Ra is over 0.5 µm.[10] Ra is an important laboratory parameter based on the depth of the scratches present on a material's surface. The arithmetic average of Ra, determined by a profilometer, is commonly used to quantitatively describe surface roughness, but it does not describe the appearance of the composite resin surface.[21] Although Ra is the most used parameter for describing roughness in the literature, it does not describe the amplitude and spacing of superficial irregularities. For this reason, other parameters should be taken into account: Rq, that evaluates peaks and valleys on the surface, Rz and Ry, that minimize the possibility of error in the interpretation of the Ra parameter, identifying the distribution of peaks and valleys. Therefore, in the present study all these four parameters were analyzed.

The importance of surface roughness for the success of restorations has been well documented. Some studies claimed that when irregularities reach a value lower than 1 µm, surfaces were visibly smooth and regular.[5, 20] More in focus, Bollen *et al.* reported that a material unable of reaching and/or maintaining an Ra value below 0.2 µm *in vitro* would be susceptible to an increase in plaque accumulation and higher risk for caries and periodontal inflammation.[6] Also Takahashi *et al.* revealed that a mean roughness of 0.2 µm is the critical threshold value for bacterial retention.[22] Although the well known clinical importance of the finishing and polishing procedures, no consensus has been established on the effectiveness of different systems used to perform an ideal finishing and polishing of the resin composite. Our study compared different finishing and polishing systems in order to improve the knowledge on the outcomes of these procedures.

In our study, GP3 presented the lowest Ra (0.09±0.04 µm) and Rz (0.17±0.10 µm) values, which is lower than the theoretical threshold of 0.2 µm; while Rq (0.69±0.21 µm) and Ry (0.74±0.29 µm) values remain under 1 µm. GP3 has the lowest roughness values in all the parameters. The technique used in GP2 showed higher values for Ra (0.19±0.05), Ry (1.28±0.38), Rq (0.29±0.12) and Rz (1.37±0.38), when compared with GP3.

Since GP2 and GP3 differ only for the presence in GP3 of the Diamond Twinst SCL, the use of the diamond paste has allowed to obtain a smoother surface than its absence. Similarly, Ferreira *et al.* found that the finishing and polishing system with diamond paste produced the best surface roughness.[6] Based on the results, GP3 showed the highest smooth surface and highest gloss. This fact demonstrated that flexible discs of aluminium oxide (Sof-Lex XT discs) effectively remove particulate inorganic filler of the organic matrix of the surface.[18] Coarse disc grain composition allows the creation of a smooth surface and the removal of irregularity on the surface of the resin composite,[17] which explains the excellent results of the techniques including aluminium oxide discs. Therefore, this type of instrument acts for the best results, as shown by GP3 values and SEM images (Figure 3). Moreover, excellent results obtained in GP3 emerged as a consequence of the use of diamond elements such as Sof-Lex Diamond Spiral Wheels with the diamond paste application. Several studies have also evidenced that clinical polishing procedures, that have achieved better performance in terms of less roughness, are those containing diamond abrasive particles.[23] All the factors mentioned above, when applied sequentially, in order to decrease the particle sizes, induce a much lower surface roughness. These assumptions help to explain and confirm the excellent results achieved in GP3. The use of the Sof-Lex XT discs is complemented by applying the Sof-Lex Diamond Spiral Wheels, respectively, in order to induce a more uniform surface texture and a much higher gloss. The results obtained for the latter of these groups evidences that the only use of the Sof-Lex XT Disc is not sufficient to reduce the surface roughness of the resin composite.

The smoothness level of the surface has a significant impact on the gloss of resin composites.[5] ADA specifications state that, after polishing, the gloss units of resin composite range from 40–60.[21] While, Cook and Thomas reported that with a 60° measurement angle, the gloss of poor



finish is generally considered to be below 60 GU, an acceptable finish between 60 and 70 GU, a good finish between 70 and 80 GU, and an excellent finish above 80 GU. Until now, there is no agreement on the geometry of the gloss measurement. Some investigators[24] demonstrated that a 20° angle allows more sensitively measurements than a 60° angle, while others[25] claimed that a 45° angle better differentiates materials. In this study, surfaces were measured by Novo-Curve glossmeter using a geometry of 60°, comparable to the observation angle of the surface by the average person, according to ISO 2813.

Among the tested finishing and polishing systems, some differences between the groups were pointed out. In our study, GP3 showed the highest values of gloss, while GP1 the lowest, and there was no statistical differences between GP2 and GP4. However, only GP3 (64,98±11,17 GU) achieved an acceptable gloss after polishing ($P<0.05$), obtaining a suitable glossy surface on the composite resin. Therefore, the aluminum oxide paste has a fundamental role in achieving an acceptable gloss value.

Therefore, it is appropriate to evaluate surface characteristics both quantitatively and qualitatively. For qualitative characterization, SEM evaluations were performed. Scanning electron micrographs of GP3 exhibited, both at low and high magnification, the smoothest surfaces. According to the results obtained from the profilometer and glossmeter analyses, GP3 showed the lowest roughness parameters and the highest gloss values after polishing. Scanning electron images of GP2 were the only ones showing similar superficial features than GP3, even if its roughness and gloss values were different, significantly ($P>0.05$). A previous study described an important relationship between gloss and surface roughness.[26] As mentioned above, the groups showed different values and trend in surface roughness and gloss.

A negative correlation between Ra value and GU has been previously reported.[21, 27] According to our findings, among the other groups, this correlation was not clear. In fact, GP1 and GP2 showed surface roughness values not statistically significant ($P>0.05$), while gloss values highlighted statistical differences ($P<0.05$). On the other hand, even though GP2 and GP4 did not show significant differences regarding gloss results, when looking at SEM micrographs, GP2 seems to have a smoother surface than GP4. This fact seems to confirm the recent scientific literature, which shows no agreement on the correlation between roughness and gloss values. Indeed, although a previous study reported a strong correlation between Ra values and gloss units,[27] a subsequent study demonstrated that this correlation was very weak.[22] Thus, this issue remains unsolved and more knowledge is needed regarding it.

Conclusions

Following results of the present study, it can be concluded that resin composite superficial characterization in terms of surface roughness and gloss depends on the finishing and polishing systems used. Despite these results showing that higher gloss may depend on lower surface roughness, further studies are needed to confirm whether there is a real correlation between them. Many manufacturers offer different finishing and polishing systems, even if no accord has been reached on the method providing the smoothest and highest gloss surface, yet. Therefore, the present *in vitro* study is of importance for the clinician, since it does show that the studied polishing system based on a diamond paste (GP3) offers the possibility of achieving high gloss values, as well as adequate smooth and mirror-like surfaces, thus making the base for properly finalizing resin-nanocomposites restorations.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Funding.—This work was supported by funds of the Polytechnic University of Marche (GoO and AP).

Authors' contributions.—Vincenzo Tosco contributed to the idea of the research protocol, wrote the present ms and performed the evaluations and data interpretation; Monterubbianesi Riccardo performed the roughness and gloss measurements and collected all the data; Giulia Orilisi contributed to data analyses, statistical evaluations and results description; Maurizio Procaccini contributed to the research protocol and supervised the project; Simone Grandini contributed to surface roughness and gloss analysis; Angelo Putignano contributed to the research protocol, to the results analyses and writing the present manuscript; Giovanna Orsini contributed to the research plan and, wrote the present manuscript and edited it.

Acknowledgements.—The authors would like to thank Michele Carrabba DDS, PhD, Department of Medical Biotechnology, University of Siena. He contributed to the setting of profilometer and glossmeter for surface roughness and gloss parameters analysis.

History.—Article first published online: October 15, 2019. - Manuscript accepted: October 7, 2019. - Manuscript received: September 8, 2019.



Hindawi
BioMed Research International
Volume 2020, Article ID 1965818, 8 pages
https://doi.org/10.1155/2020/1965818

Hindawi

Research Article

How Can Different Polishing Timing Influence Methacrylate and Dimethacrylate Bulk Fill Composites? Evaluation of Chemical and Physical Properties

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Academic Editor: Giuseppina Nocca

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The polishing procedure is commonly performed after direct composite restorations, and little information exists regarding the right timing during which it should be performed on bulk fill composites. This study investigated the effect of polishing timing on the degree of conversion (DC), Vickers microhardness (VMH), and surface morphology of a methacrylate- (MET-) and dimethacrylate- (DMET-) based bulk fill composite, by using FT-NIR, microhardness tester, and SEM. Composite samples were divided as follows: in Group I (immediate), samples were polished immediately after curing (t_0); in Group D (delayed), samples were polished after 24 h from curing (t_{24}), whereas the unpolished samples were considered as controls (Group C). The DC and VMH values were evaluated before and after polishing, at t_0 and t_{24}. Statistical analysis was performed with a significance level set at $p < 0.05$. At t_0, DC increased after polishing in both tested composites ($p < 0.05$), while at t_{24}, Group I and Group D were not different. By considering VMH, in the case of MET, all groups were not different both at t_0 and t_{24}. On the other hand, at t_0, VMH values of DMET increased after polishing. At t_{24}, DMET Group I and DMET Group D were not different. Qualitative evaluations of scanning electron micrographs showed that the surface morphology of MET presented a more irregular aspect than the DMET one. In summary, since the immediate polishing of MET can improve the DC, without negatively affecting VMH, but showing an irregular surface, it is suggested to wait 24 hours before proceeding with polishing. Otherwise, for DMET, the immediate polishing could definitively be recommended, since it improves both DC and VMH, also producing a regular surface. Therefore, clinicians may always safely polish a restoration performed using DMET-based bulk fill composites in one-chair appointment, avoiding a second appointment.

1. Introduction

The light polymerization of dental composites leads to crosslinked networks between monomers inside the resin composites [1]. The formation of these crosslinked networks, evaluated by the degree of conversion (DC), together with the Vickers microhardness (VMH) of materials can have great importance in the success and longevity of dental restorations [2, 3]. It is well known that materials with low DC and VMH enhance the failure rate of reconstruction: a low DC can cause a progressive worsening of the superficial aspect or low biocompatibility, while a low VMH can be linked with low wear resistance and scratches on the reconstruction surface [2, 4]. Moreover, the same DC of different materials can give different VMH [5, 6]. In clinical practice, after the curing of resin composites, clinicians should finish and polish them in order to remove excess of material, fit the occlusion, obtain a smooth surface, and improve their aesthetics [7].

Finishing indicates the contouring or reduction of restoration to obtain ideal anatomy. Polishing reduces the



roughness created by finishing [8, 9]. The roughness and an irregular surface lead to plaque accumulation, gingival inflammation, superficial staining, and secondary caries: all of these are some of the frequent reasons for tooth reconstruction failures. Then, an appropriate finishing and polishing are critical clinical procedures that enhance the longevity of restorations [10]. However, polishing and finishing effects depend on the type of resin composites and can influence their chemical and physical properties [11].

In the last few years, the so-called bulk fill composites have been introduced in the market: they consist of a combination of new chemical monomers (methacrylate or dimethacrylate) and fillers with an enhancement of their translucency and, consequently, with the potential of gaining an optimal DC [10, 12]. Although there are many articles on the effects of polishing on traditional resin composites [8, 13], there are no detailed studies concerning the timing effects of the new polishing systems on the properties of bulk fill composites.

Therefore, the aim of this study was to improve the knowledge on these new bulk fill composites, evaluating the effects of immediate and delayed polishing on the DC and VMH of methacrylate and dimethacrylate-based bulk fill composites. The null hypotheses were as follows: (1) the timing of the polishing does not influence the DC of the tested bulk fill composites; (2) the timing of the polishing does not influence their VMH.

2. Materials and Methods

The following two bulk fill composites were used: a low-viscosity composite, Estelite Bulk-Fill Flow shade A2 (Tokuyama Dental Inc., Encinitas, CA), which is a methacrylate-based composite (MET), and a high-viscosity composite, Filtek One Bulk Fill Restorative shade A2 (3M, St. Paul, MN, USA), a dimethacrylate-based composite (DMET). Their composition and characteristics are shown in Table 1. For each composite, ten samples were prepared by using homemade Teflon cylinders (3.0 mm in height and 6.0 mm in internal diameter) (Figure 1). All samples were photopolymerized in bulk using Elipar DeepCure S lamp (3M, St. Paul, MN, USA) for 20 sec with an irradiance around 1470 mW/cm^2 and a spectrum range between 430 and 480 nm. During the photopolymerization, samples were covered with a Mylar strip to exclude oxygen inhibition of surface layer. After curing, the samples were randomly divided into the following groups of five samples for each: MET Group I (I: immediate) and DMET Group I, in which the samples were finished and polished immediately after curing (t_0); MET Group D (D: delayed) and DMET Group D, in which the other samples were finished and polished after 24 h (t_{24}) from curing. The unpolished samples of Group I at t_0 and Group D at t_0 and t_{24} were considered as controls (MET Group C and DMET Group C). Measurements of DC and VMH were performed for all the samples before and after each finishing and polishing procedure, at both t_0 and t_{24}. Two different disks covered by aluminum oxide (Sof-Lex Disks, 3M, St. Paul, MN, USA) were used to finish the samples: a final contouring medium disk (30 μm abrasive particle size) and a finishing fine disk (14 μm abrasive particle size). The different disk sizes were used in order to simulate the effect of finishing burs which can be used both in anterior and posterior reconstructions. Successively, elastomer wheels impregnated with aluminum oxide and diamond particle (Sof-Lex™ Diamond Polishing System, 3M, St. Paul, MN, USA) were used for the polishing procedures, they consist of a beige prepolishing spiral wheel and a pink high-polishing spiral wheel. Each Sof-Lex disk was used in a circular motion applying light pressure for 10 sec with a slow speed handpiece at 7500 rounds/min. Then, the beige and pink spiral wheels were used with a slow speed handpiece at 6000 rounds/min; a diamond paste (Shiny paste, Enamel Plus, Genova, Italy) was used only with the pink spiral wheel. All the procedures were performed by a single dental operator to minimize operator changes in variability. Each finishing and polishing instrument was discarded immediately after use, and before the evaluations, the samples were washed with distilled water and dried. The samples were stored at 37°C.

2.1. Degree of Conversion Analysis. A Perkin Elmer Spectrum One NTS FT-NIR spectrometer equipped with a FR-DTGS detector was used for DC evaluation [5, 14]. For this purpose, on the top of each sample, spectra were acquired in the reflection mode in the 10000–4000 cm^{-1} spectral range, by using the Near Infrared Reflectance Accessory (NIRA) (Perkin Elmer). All the spectra were interpolated, and two-points baseline linear fitted in the 6300–5300 cm^{-1}, and the height of the bands at 6165 cm^{-1} and 5990 cm^{-1} was calculated (Spectrum 10.4 software package, Perkin Elmer). The band at 6165 cm^{-1}, corresponding to the alkene carbon-carbon double bond vibration (band B), decreased during the polymerization process, while that at 5990 cm^{-1}, corresponding to the vibrational modes of the aromatic benzene ring, did not change in intensity (band A). For each spectrum, the ratio between the heights of band B and band A was calculated (band height ratio B/A) [15]. To evaluate the DC, calibration curves were plotted assuming that the ratio B/A of the no cured material may represent the 0% of polymerization, while the same ratio at 1 week, may be taken as 100% of polymerization [5, 16].

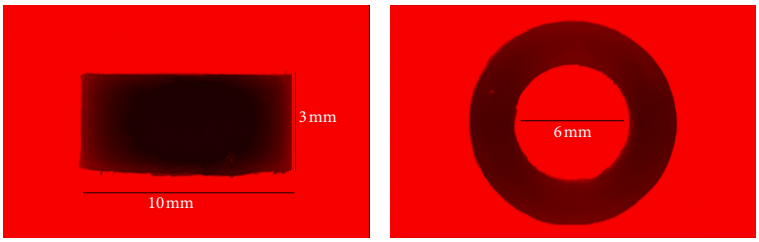
2.2. Microhardness Analysis. On the same surface of the samples analyzed by FT-NIR spectroscopy, the VMH was determined by using the Leitz microhardness tester (Wetzalr GMBH, Wetzlar, Germany) [14]. The method consisted of indenting the sample by using a pyramid-shaped diamond with a load of 50 g for 15 sec. For each sample, three measurements were performed, respectively, in the middle of the sample, at 0.15 mm and 0.3 mm from the center. After removing the load, the values of the two indentation diagonals were evaluated by using a microscope; the area of the sloping surface was obtained and used to determine the corresponding hardness value. Calculations were made by using Hardness-Course Vickers/Brinell/Rockwell version 10.4.4 software package [17–19].



Table 1: Composition of the resin-based composites used in this study.

Type	Brand	Composition	Filler load (%)
Bulk fill low-viscosity methacrylate composite	Estelite Bulk-Fill Flow (Tokuyama Dental)	Bis-GMA, Bis-MPEPP, TEGDMA, 200 nm spherical silica, and zirconia SiO_2–ZrO_2 200 nm spherical SiO_2–ZrO_2	70.0 wt 56.0 vol
Bulk fill high-viscosity dimethacrylate composite	Filtek™ One Bulk Fill Restorative (3M ESPE)	AFM, AUDMA, UDMA, DDDMA, nonagglomerated/nonaggregated 20 nm silica filler, a nonagglomerated/ nonaggregated 4 to 11 nm zirconia filler, an aggregated zirconia/silica cluster filler (comprising 20 nm silica and 4 to 11 nm zirconia particles), and an YbF_3 filler consisting of agglomerate 100 nm particles	76.5 wt 58.5 vol

w, weight percentage; vol, volume percentage; AFM, addition-fragmentation monomer; AUDMA, aromatic urethane dimethacrylate; Bis-GMA, bisphenol A glycidyl dimethacrylate; Bis-MPEPP, bisphenol A polyethoxy methacrylate; DDDMA 12-dodecane-dimethacrylate; DMA, dimethacrylate; TEGDMA, triethylene glycol dimethacrylate; UDMA, urethane dimethacrylate; YbF3, ytterbium trifluoride.



(a) (b)

Figure 1: Pictures of homemade Teflon cylinders: frontal (a) and upper (b) view. Height = 3 mm; outside diameter = 10 mm; inside diameter = 6 mm.

2.3. Surface Evaluation. A Field Emission Scanning Electron Microscope Zeiss Supra 40 (SEM), using a power of 3000 kV and a ×400 magnification, was used for a further surface evaluation of all groups of composites, before and after polishing. The irregularity of the surface was evaluated by using the native software of SEM.

2.4. Statistical Analyses of DC and VMH Data. Statistical analysis was performed with R Project for Statistical Computing 3.3.0 (https://www.r-project.org/) and Microsoft Excel 2013. Normality of data distribution and homogeneity of group variances were verified by the Kolmogorov–Smirnov test and the Levene test, respectively. Given the normality and homogeneity of the distribution, one-way analysis of variance (one-way ANOVA) and Tukey's test for comparisons between groups were chosen ($p < 0.05$). The Pearson test was used to evaluate the correlation between the DC and VMH of each material type.

3. Results

The statistical evaluations of DC and VMH are reported in Figures 2 and 3, respectively. At t_0, the DC of MET Group C (69.68 ± 3.90) was significantly different from MET Group I (74.88 ± 2.06) ($p = 0.03$); at t_{24}, the difference between the values of all the groups was not significant (MET Group C: 95.53 ± 4.01; MET Group I: 91.73 ± 5.21; MET Group D: 94.21 ± 2.43) (Figure 2(a)). Moreover, all DC MET values of t_{24} were different than t_0 values.

On the other hand, at t_0, the DC of the DMET Group C (81.70 ± 2.63) was different from that of DMET Group I (89.57 ± 3.62) ($p = 0.03$); at t_{24}, the difference between the values of all groups was not significant (DMET Group C: 91.78 ± 1.48; DMET Group I: 92.48 ± 4.02; DMET Group D: 91.96 ± 2.62) (Figure 2(b)). Therefore, the DC values of all groups at t_{24} were not different from DMET Group I at t_0. However, at t_{24}, the DC of all polished groups of both composites was not different.

By considering VMH, in MET, both at t_0 and t_{24}, the difference between the DC values of all groups was not significant (Figure 3(a)). About DMET, at t_0, the VMH values of DMET Group I (88.43 ± 6.17) resulted significantly higher than DMET Group C (81.01 ± 1.01) ($p = 0.02$). At t_{24}, DMET Group I (90.9 ± 3.20) was not significantly different from DMET Group D (85.03 ± 1.98). Moreover, DMET Group I at t_{24} was not different from DMET Group I at t_0 (88.43 ± 6.17).

No strong correlation was found between DC and VMH, both in MET (correlation value: 0.16) and DMET (correlation value: 0.40).

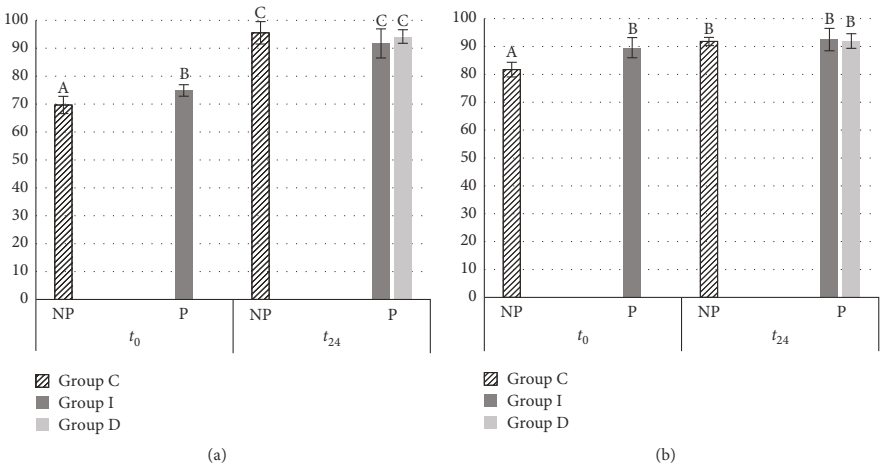


Figure 2: Degree of conversion values calculated at t_0 and t_{24}, using different polishing timing for (a) Estelite Bulk-Fill Flow (MET) and (b) Filtek One Bulk Fill (DMET) composites. Tukey test was used, and different superscript letters (A, B, and C) represent statistically significant difference ($p < 0.05$). NP: the samples were not polished; P: the samples were polished; Group I: the samples were cured, finished, and polished immediately after curing; Group D: the samples were cured at t_0 but finished and polished after 24 hours; Group C: the samples were not polished and were used as controls.

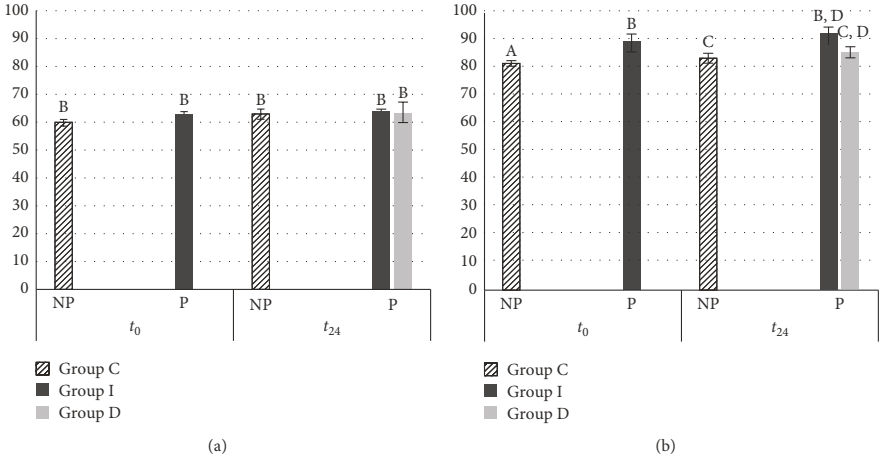
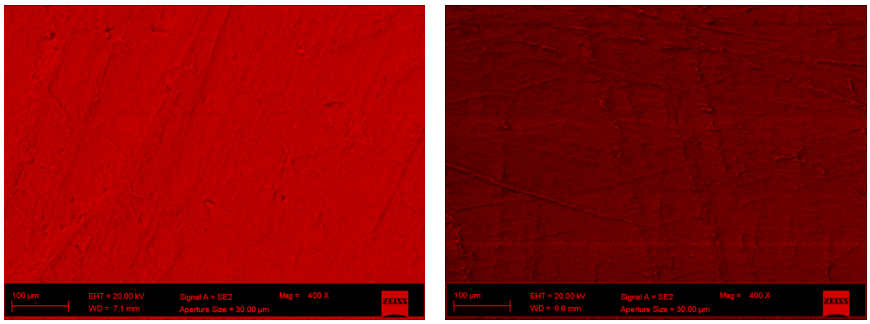


Figure 3: Vickers microhardness calculated at t_0 and t_{24}, using different polishing timing for (a) Estelite Bulk-Fill Flow (MET) and (b) Filtek One Bulk Fill (DMET) composites. Tukey test was used, and different superscript letters (A, B, and C) represent statistically significant difference ($p < 0.05$). NP: the samples were not polished; P: the samples were polished; Group I: the samples were cured, finished, and polished immediately after curing; Group D: the samples were cured at t_0 but finished and polished after 24 hours; Group C: the samples were not polished and were used as controls.

SEM results showed that, in the tested materials, different surface patterns may be detected. In MET, the low-viscosity resin composite, both Groups I and D showed an irregular surface (Figures 4(a) and 4(b)). On the other hand, DMET Group I showed a surface smoother and more regular than DMET Group D (Figures 5(a) and 5(b)).

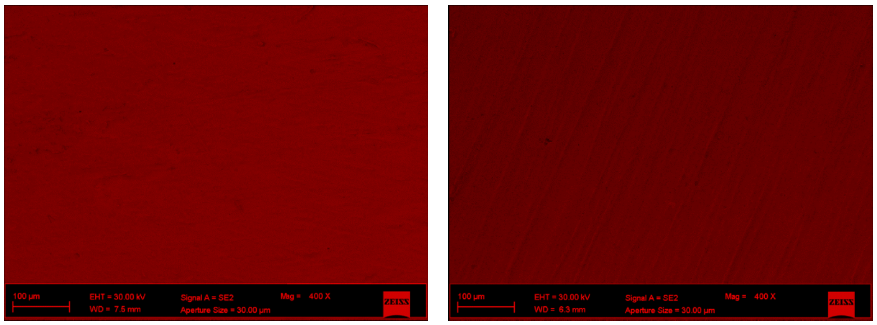
4. Discussion

It is well known that the properties of resin composites are material dependent [2, 5, 8]. In clinical practice, considering the oxygen inhibition on the surface, finishing and polishing procedures are important for improving DC and VMH of



(a) (b)

Figure 4: Scanning electron microscope images (400x magnification) of Estelite Bulk-Fill Flow: (a) MET Group I: polished immediately after curing; (b) MET Group D: polished after 24 hours from curing.



(a) (b)

Figure 5: Scanning electron microscope images (400x magnification) of Filtek One Bulk Fill Restorative composite: (a) DMET Group I: polished immediately after curing; (b) DMET Group D: polished after 24 hours from curing.

dental composites, resulting in the success of a restoration [20]. Moreover, finishing and polishing steps are used to modify and improve the profile of a direct composite reconstruction: its success depends on both the type of composite used and the system adopted to finalize it [11, 20]. Although a few reports have investigated the chemical-physical properties of composites after using modern finishing and polishing systems, no studies have evaluated the timing effect of the new polishing system on bulk fill composites containing methacrylate and dimethacrylate monomers [10, 13, 21–23].

In the present study, the Mylar strip was used in order to avoid the oxygen inhibition of the surface layer. DC and VMH of tested composites were evaluated after performing the polishing procedures at different time points. In summary, for DMET the polishing procedure helped the resin composite to reach the same DC recorded at t_{24}, immediately after curing. On the other hand, for MET, the polishing procedures increase the DC at t_0 even if it did not reach the t_{24} values. As hypothesized by the scientific literature, this phenomenon could be explained by the fact that brushing, during polishing, could improve DC due to the heat, generated by the rotary instruments which catalyze the polymerization process on the surface layer [24]. However, the authors speculate that the DC difference between MET and DMET could be based on their monomer combination type: DMET has an addition-fragmentation monomer, which can promote the increase of DC better than methacrylate monomers, during polishing.

Another important property concerning the dental composite and its clinical use is the hardness. It describes the deformation degree of the material, and it is a valuable parameter for making comparison between tooth structure and masticatory forces, especially in the posterior stress-bearing areas [25], thus being considered one of the most important properties. Using materials possessing hardness similar to that of dentin is mandatory to achieve an optimum clinical restoration performance, and this holds mainly for bulk fill composites [26]. Nowadays, clinicians often use bulk fill composites in posterior areas, where their placement is difficult and occlusal forces are more relevant compared to anterior areas [26].

Our results revealed that the hardness of the tested composites, mainly in case of DMET composite, improves



after polishing: VMH values of DMET Group I increased after polishing ($p < 0.05$), and they were not statistically different from DMET Group D at t_{24} ($p > 0.05$). Regarding VMH of all MET groups, the values were not statistically different ($p > 0.05$). Some authors have hypothesized that, immediately after curing, the surface layer, mostly composed of the organic matrix, may further polymerize during polishing, thus increasing the resistance of the surface layer [13, 27]. However, in our findings, the two tested composites are different not only for the monomers type, but even in vol% and wt%, as well as in filler dimensions. DMET contains a thicker filler (DMET: 4–20 nm versus MET: 200 nm) and a higher wt% and vol% than MET, and consequently, it contains more filler surface to be linked by the matrix monomers and silanes. Maybe, the friction temperature of the polishing procedure could enhance the silanes action [28–30]. The temperature could increase the ability of silanes to link the filler and matrix, improving the hardness. Then, DMET, with a higher filler surface area (more vol% and small filler size), which can link the silanes, could be more influenced by the temperature than MET. Although Chinellatti et al [31] proved that delayed finishing and polishing procedures generally result in a surface similar to or even harder than the one obtained with immediate finishing and polishing procedures, we underline that, in their study, they used flowable, microfilled and minifilled composites, and no bulk fill composites were evaluated. Partially in disagreement with our results, Cenci et al [22] assessed that the polishing procedure, immediately after polymerization, causes incomplete curing and that the stress generated by using rotary instruments may influence the integrity of the restoration, being the range of filler particles a possible explanation for this discrepancy. However, their study dealt only with microfilled or microhybrid resin composites, whereas neither bulk fill composites, specific composite monomers, nor nanofilled composites were evaluated.

In clinical practice, especially in the posterior area, after checking occlusal contacts, clinicians seldom leave composite restorations unfinished and unpolished. It is therefore important to evaluate the occlusal surface of composite materials using various methods, including SEM [32].

The performance of finishing and polishing procedures are often verified using scanning electron micrographs, by qualitatively evaluating shape and contour changes that may not be observed with a profilometer (which gives an objective and quantitative evaluation) [33, 34]. In our study, the characteristics of the tested composites appear different. SEM pictures of both MET groups show more irregular surfaces (>0.2 μm) than DMET ones (Figures 4 and 5), mainly when polishing was performed at t_0.

The formation of an irregular surface on resin composites depends on several material factors, such as the type, shape, size, and distribution of the inorganic filler, and can increase the risk of plaque formation [35–38]. In our case, the presence of thinner filler size results in a reduced matrix interparticle spacing, meaning more protection of the organic matrix and a reduced filler grabbing (as in DMET, with a higher ratio of nanofiller/organic matrix): more organic matrix than filler influences the surface of resin composite because the matrix, less hard than the filler, can show some irregularities after polishing.

Finally, as the previous traditional composites, in both MET and DMET resin composites, no correlation between DC, and VMH was found. It means that not only the DC but also the resin composition influences the mechanical properties of the new bulk fill composites. Although our study presents several limitations, such as the fact that the investigated materials were different in consistency, type of matrix (MET/DMET), and fillers (differently sized particles), it is the first report on the influence of polishing timing on chemical and mechanical properties of the methacrylate- and dimethacrylate-based bulk fill composites.

Therefore, it would be interesting to plan future studies in which other similar high-viscosity bulk fill composites will be analyzed, comparing the correlation among differences in resin monomers and particles content. In particular, the nanotechnological impact of aggregating miscellaneous particles with nanometric dimensions will be taken into account in correlation with the various recently developed monomeric combinations, in order to better understand which is the influence of MET/DMET changes that really improve the clinical performance of these materials.

In summary, the first hypothesis can be rejected. In Group I of both composites, polishing procedures increase the DC at t_0, reaching, mainly in DMET, the same DC of t_{24}. Furthermore, the second hypothesis can be partially rejected. In the DMET group, the polishing procedure leads to an improvement of VMH, both at t_0 and t_{24}. Noteworthy is that at t_{24}, no difference is evident between immediate and delayed polishing of DMET groups. In MET, polishing procedures do not affect VMH values in both groups, whereas DC seems always to be improved.

Our finding may be applied for guiding the clinical use of the above-tested high- (DMET) and low- (MET) viscosity bulk fill composites. In the case of MET, although the immediate polishing improves the DC and VMH is not affected, we recommend waiting 24 hours before polishing, because the surface is more regular in delayed than in immediate polishing. On the other hand, the immediate polishing of restorations performed using DMET could definitively be recommended, since both DC and VMH were increased and a smooth and regular occlusal surface was also observed. Thus, clinicians may always perform and finalize their restorations in one single appointment, saving time, without compromising the good physical properties of these composite resin-based materials.

Data Availability

The present data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.



Authors' Contributions

Riccardo Monterubbianesi and Vincenzo Tosco contributed equally to this work.

Acknowledgments

The authors are grateful to Prof. Giorgio Tosi for the precious contribution in the study design. Moreover, the authors thank the SIMAU for the instrumental support and mainly the assistance provided by Prof. Elisabetta Giorgini and Adriano Di Cristoforo.

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Odontology
https://doi.org/10.1007/s10266-020-00558-0

ORIGINAL ARTICLE



Comparison of two curing protocols during adhesive cementation: can the step luting technique supersede the traditional one?

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Received: 19 September 2019 / Accepted: 23 September 2020
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Abstract
This study aims to compare the degree of conversion of two different curing protocols used during adhesive cementation. The following resin luting agents were tested: Hri Flow (MF) and pre-heated Hri Micerium (MH); light-cure Nexus Third Generation (NX3L) and dual-cure Nexus Third Generation (NX3D); dual cured RelyX Ultimate (RXU) and light-cure RelyX Veneers (RXL). For each tested material, ten samples were prepared and divided into two groups which had different curing protocols (P1 and P2): in P1, samples were cured for 40 s; in P2, samples were cured for 5 s, and then, after 20 s, cured again for additional 40 s. The degree of conversion (DC) was evaluated both during the first 5 min of the curing phase and after 1, 2, 7, 14 and 28 days ($p = 0.05$). Different trends were observed in DC values after 5 min by comparing P1 and P2. In both P1 and P2, DC decreased as follows, MH > MF > NX3L > RXL > RXU > NX3D. There were significant differences of DC values among all resin luting agents ($p < 0.05$) in P1, while no significant differences existed between MH and MF, and NX3L and RXL in P2. At 1, 2, 7, 14 and 28 days the light curing luting agents had a higher DC than the dual luting agents ($p < 0.05$). P1 and P2 were not statistically different at each time point ($p > 0.05$). Both P1 and P2 protocols let achieve an acceptable DC after 28 days. The tested P2 can be safely used to lute indirect restorations, simplifying the removal of cement excesses.

Keywords Curing protocols · Resin luting agents · Degree of conversion · Kinetics of conversion degree · NIR spectroscopy

Introduction

The development of reliable adhesive systems between the tooth and resin-based materials (RBM) has led to more conservative dental treatments. Before the introduction of modern adhesive materials, dentists prepared teeth to have mechanical retention, and in doing so, often sacrificed healthy dental tissue. The age of adhesive cementation has led to minimally invasive dentistry, in which the clinician can lute indirect restorations using resin luting agent thereby preserving dental tissue.

Resin luting agents are intermediates between the tooth substrate, with or without a bonding agent, and the indirect restoration. They can be divided into two categories: traditional resin-based composites and resin cements. These latter can be divided into light, self or dual cured according to the curing procedure, depending on own monomers and formulation.

A free radical reaction allows resin luting agents to move from a viscous to a rigid state in a process called polymerization. During the curing process, the terminal aliphatic C = C bonds are broken and converted into primary C–C covalent bonds between methacrylate monomers, the ratio of this conversion is described with the degree of conversion (DC). However, the formation of free radical varies with the activator system [1].

Vincenzo Tosco and Riccardo Monterubbianesi equal contribution.

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Published online: 31 October 2020



Multiple factors can influence the DC of luting resin agents such as their monomer content, the components of the activation system, and the type of polymerization. Moreover, the level of DC achieved during the polymerization directly influences the physical and mechanical properties of resin luting agents, as the whole RBM [2], therefore, affects the longevity of the indirect restoration [3]. Inferior mechanical properties, greater discoloration and degradation are the main drawbacks of a low DC, resulting in a resin luting agent with poor wear resistance and poor colour stability [4, 5].

Because during clinical application, the thickness of the indirect restoration can reduce the amount of curing light that reaches the resin luting agent, then the type of curing becomes a fundamental factor for the success of a restoration [6, 7]. For example, with a thick inlay/overlay restoration or with deep cavities, clinicians are uncertain whether the resin luting agent have been properly polymerized. Moreover, removing the excesses of resin luting agents could be difficult and time-consuming, mainly in the interdental space, because their hardening.

For this reason, during the luting phase, some clinicians adopt a step curing technique to fix the indirect restoration allowing the clinician to remove the soft excess of material around the indirect restoration. There are no scientific articles or evidences about the chemical stability after this kind of "step luting" procedure. Then, improving the knowledge on polymerization kinetics could be relevant, particularly during the cementation of an indirect restoration.

The aim of this study was to analyse the effects caused by two different curing protocols (P1 and P2) on the polymerization of various resin luting agents. The two null hypotheses were: 1) All the tested materials have the same DC; 2) The curing protocols do not affect the DC of tested materials.

Materials and methods

Samples preparation and FT-NIR analysis

The following resin luting agents were investigated: the light-cure flow resin composite, Enamel Plus HRi Flow, shade UD3 (MF) (Micerium, Avegno, Genova, Italy); the pre-heated light-cure high viscosity resin composite, Enamel Plus HRi, shade UD3 (MH) (Micerium, Avegno, Genova, Italy); the light-cure resin cement, Nexus Third Generation, shade Yellow (NX3L) (Kerr, Orange, CA, USA); the dual-cure resin cement, Nexus Third Generation, shade Yellow (NX3D) (Kerr, Orange, CA, USA); the light-cure resin cement, RelyX Veneer, shade A3 (RXL) (3M ESPE, St. Paul, MN), and the dual-cure resin cement, RelyX Ultimate, shade A3 (RXU) (3M ESPE, St. Paul, MN). The pre-heated composite MH was obtained by heating a compule for 10 min at 55 °C in the oven (Ena Heat, Micerium, Avegno, Genova, Italy), following the manufacturer instruction. The composition of the tested materials is described in Table 1.

Each tested material was placed on a Kaltek Glass (1.1 mm thick) inside a thin Teflon Ring (0.2 mm height and 15.0 mm internal diameter) and covered by another thin glass (0.2 mm thick), to obtain a disk-shape of the material with a diameter of 15.0 mm and a thickness of 0.2 mm (Fig. 1). Samples were divided into two groups and then submitted to the following curing protocols: five samples were cured for 40 s (P1); the other five samples were cured for 5 s, and then, after 20 s, cured again for additional 40 s (P2). The sample size was calculated using MATLAB (version 7.5.0, MathWorks, Natick, MA, USA) and by the analysis of previous studies [8–11]. An Elipar DeepCure S light (3 M Espe, Seefeld, Germany) was adopted, with an irradiance of 1470 mW/cm^2 ± 20% and a spectrum range between 430 and 480 nm. During curing phases, a polymerized composite disk (Filtek Supreme A3B Plus, 3M) of 2.0 mm of thickness and 25.0 mm of diameter was interposed between the tip of the lamp and the resin cement sample (Fig. 1). The composite disk was removed after each curing phase. The translucency of the composite disk was 10.07; it was calculated using the CIELab space as the difference in colour between the disk as it appeared against the standard white background and as it appeared against the standard black background, according to the following equation:

$$TP = \left[(LW-LB)^2 + (aW-aB)^2 + (bW-bB)^2\right]^{1/2}$$

All the values were evaluated by SpectroShade-Micro (MHT S.p.a., Verona, Italy) on white (W) and black (B) background: L* (lightness, where 100 represents white and 0 represents black), a* (red-green chromatic coordinate) and b* (blue-yellow chromatic coordinate).

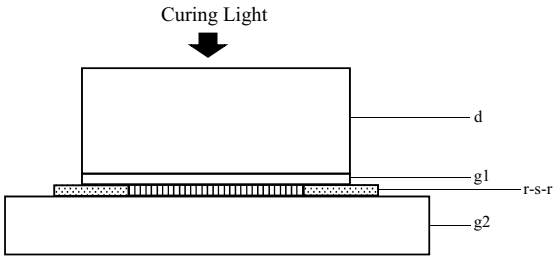
The kinetic evaluation of the polymerization process of all samples was performed by a Perkin Elmer Spectrum One NTS FT-NIR spectrometer, operating in the 10000–4000 cm^{-1} spectral range. The NIR spectra of unpolymerized materials were first purchased (T0). Then, samples were cured following the appropriate curing protocol (P1 or P2) and the NIR spectra were collected in continuous mode for 5 min (one every 13 s) using the TimeBase software package (PerkinElmer). The NIR spectra of the same samples were also collected after 1, 2, 7, 14 and 28 days. In the waiting time, samples were stored in dry and dark conditions at room temperature. All the collected spectra were interpolated in the 7000–4000 cm^{-1} spectral region and 2-points baseline corrected.



Table 1 Composition of resin luting agents

Code	Brand (Shade)	Manufacturer	Type	Composition	Filler composition
MF	Enamel Plus HRi Flow (UD3)	Micerium, Avegno, Genova, Italy	Light-Cure flow resin composite	BisGMA, BDDMA, UDMA, glass filler highly dispersed SiO_2	77% wt Unknown%vol
MH	Enamel Plus HRi (UD3)	Micerium, Avegno, Genova, Italy	Pre-heated Light cure resin composite	DiUDMA, BisGMA, BDDMA Filler: Particles of zirconium oxide and glass	80 wt% 63 vol%
NX3L	Nexus Third Generation (Yellow)	Kerr, Orange, CA, USA	Light-Cure resin cement	Uncured Methacrylate Ester Monomers, minors filler, pigments, radio-paque agent 20–40%	63 wt % 38 vol%
NX3D	Nexus Third Generation (Yellow)	Kerr, Orange, CA, USA	Dual-cure resin cement	Uncured Methacrylate Ester Monomers 20–40%	Unknown%wt 47 vol%
RXL	RelyX Veneer (A3)	3M ESPE, St.Paul, MN	Light-Cure resin cement	TEGDMA/BisGMA; Parti-cles of zirconia/silica and colloidal silica	66. 0 wt % 47.0 vol%
RXU	RelyX Ultimate (A3)	3M ESPE, St.Paul, MN	Dual-cure resin cement	Methacrylate monomer, alkaline filler, initiator components, stabilizers, pigments, rheological additives, fluorescence dye	67.0wt % 43.0 vol %

Bis-GMA bisphenol-A glycidyl methacrylate, *BDDMA* 1,4-butandioldimethacrylate, *TEGDMA* triethylene glycol dimethacrylate, *UDMA* urethandimethacrylate



d: Composite disk, height(h)=2.0 mm; g1: Thin glass, h=0.2 mm; r: Teflon ring, h=0.2 mm; s: Sample h=0.2 mm; g2: Kaltek Glass, h=1.1 mm.

Fig. 1 Schematic view of the assembled device for sample polymerization. d: Composite disk, height(h) = 2.0 mm; g1: Thin glass, h = 0.2 mm; r: Teflon ring, h = 0.2 mm; s: Sample h = 0.2 mm; g2: Kaltek Glass, h = 1.1 mm

DC evaluations

The DC of the resin luting agents was calculated by comparing the height of the following peaks in the un-polymerized and polymerized samples: near 6166 cm^{-1} (RXU, RXV, NX3D and NX3F samples) and near 4744 cm^{-1} (MH and MF samples) (related to the C = C moiety directly involved in the polymerization; bands A); near 5993 cm^{-1} (RXU, RXV, NX3D and NX3F samples) and near 4620 cm^{-1} (MH and MF samples) (Spectrum 10.4 software package, Perkin Elmer). For MH and MF, different NIR peaks were chosen due to the more convoluted spectral profile because their specific composition. For each spectrum, the ratio between the heights of B and A bands was calculated (B/A), and then converted in DC using a calibration curve [12, 13].

Statistical analysis

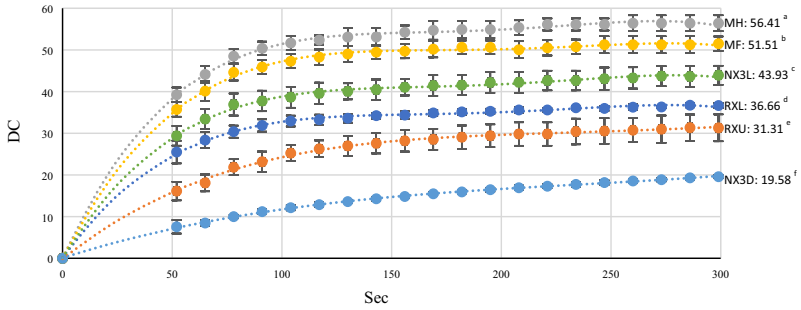
After normality and homogeneity evaluations of the data, One-way ANOVA was performed for DC changes, within



each protocol. The Tukey HSD test was used for multiple comparisons between groups. Student's t-test was used for the comparisons between the different protocols of the same materials and time points. All tests were performed with $p < 0.05$, using the statistical package Data Analysis in Microsoft Excel 2013 and R Project.

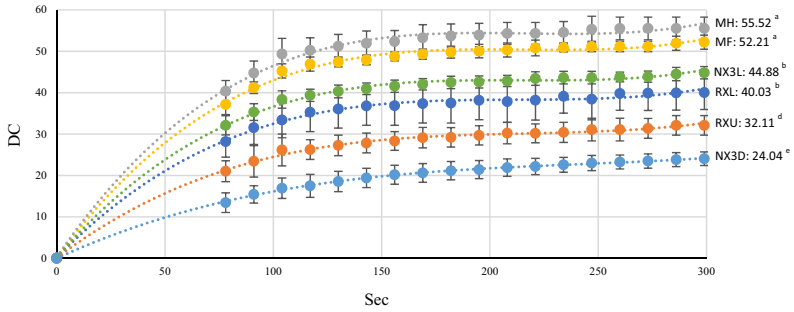
Results

During the first 5 min, all the tested materials showed an increasing exponential trend for DC values for both P1 and P2 protocols. Moreover, there were significant differences of DC values among all resin luting agents ($p < 0.05$). At the 5-min time point, different DC values were obtained both in relation to the tested materials and the protocols used; in particular, the following significant decreasing order were found: for P1, MH > MF > NX3L > RXL > RXU > NX3D ($p < 0.05$) (Fig. 2); for P2, MH > MF > > NX3L > RXL > RXU > NX3D; however, there were significant differences of DC values among the resin luting agents except between MH and MF, and NX3L and RXL ($p < 0.05$) (Fig. 3). A statistically significant difference between the two protocols was detected only for NX3D (P1, 19.58 ± 0.37 *vs* P2, 24.04 ± 1.64; $p = 0.004$), while MH, MF, NX3L and RXU were not statistically different ($p < 0.05$). The DC values calculated at days 1, 2, 7, 14 and 28 are listed in Table 2. On day 1, MF (73.83 ± 1.61), MH (75.59 ± 2.73) and NX3L (72.73 ± 4.04) had the highest DC in P1 and MF (74.91 ± 0.99), MH (74.16 ± 2.82) in P2, while the lowest



MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate; DC, Degree of Conversion; Sec, seconds.

Fig. 2 Trend of the Degree of Conversion evaluated in the first 5 min for the tested materials P1. Different superscript letters indicate statistically significant differences ($p < 0.05$)



MF, Enamel Plus HRi Flow; MH, pre-heated Enamel Plus HRi; NX3L, NX3 light-cure; NX3D, NX3 dual-cure; RXL, RelyX Veneer; RXU, RelyX Ultimate; DC, Degree of Conversion; Sec, seconds.

Fig. 3 Trend of the Degree of Conversion evaluated in the first 5 min for the tested materials P2. Different superscript letters indicate statistically significant differences ($p < 0.05$)



Table 2 Degree of Conversion evaluated for the tested resin materials after 1, 2, 7, 14 and 28 days

		Day 1	Day 2	Day 7	Day 14	Day 28
MF	P1	73.83 ± 1.61^{a}	78.50 ± 1.01^{a}	79.41 ± 2.12^{ac}	85.97 ± 2.00^{a}	89.16 ± 3.24^{a}
	P2	74.91 ± 0.99^{1}	79.36 ± 0.30^{1}	79.66 ± 1.24^{1}	87.82 ± 0.97^{1}	87.07 ± 1.47^{1}
MH	P1	75.59 ± 2.73^{a}	79.68 ± 1.98^{a}	85.77 ± 2.59^{a}	86.64 ± 2.58^{a}	92.91 ± 3.13^{a}
	P2	74.16 ± 2.82^{1}	$78.09 \pm 6.63^{1,2}$	85.35 ± 1.06^{2}	87.20 ± 1.49^{1}	92.75 ± 1.73^{2}
NX3L	P1	72.73 ± 4.04^{a}	76.76 ± 4.66^{a}	86.42 ± 4.57^{a}	90.56 ± 3.81^{a}	92.27 ± 5.19^{a}
	P2	66.42 ± 5.60^{2}	76.28 ± 1.95^{2}	85.75 ± 2.50^{2}	88.83 ± 2.69^{1}	93.57 ± 2.17^{2}
NX3D	P1	52.24 ± 0.80^{b}	58.73 ± 1.29^{b}	72.09 ± 1.12^{b}	78.08 ± 1.57^{b}	82.94 ± 0.85^{b}
	P2	52.97 ± 1.32^{3}	58.19 ± 1.25^{3}	72.85 ± 0.76^{3}	78.89 ± 1.06^{2}	84.03 ± 0.61^{1}
RXL	P1	63.15 ± 1.81^{c}	68.37 ± 1.37^{c}	86.64 ± 2.52^{a}	90.02 ± 3.39^{a}	92.51 ± 1.98^{a}
	P2	$61.98 \pm 2.07^{2,4}$	$69.28 \pm 7.80^{2,4}$	$81.87 \pm 3.06^{1,2}$	85.56 ± 5.24^{1}	$89.45 \pm 4.12^{1,3}$
RXU	P1	61.27 ± 6.87^{c}	64.56 ± 7.38^{c}	73.65 ± 7.88^{cb}	79.65 ± 11.42^{b}	86.18 ± 10.60^{a}
	P2	$58.47 \pm 4.96^{2,4}$	$62.23 \pm 5.27^{3,4}$	71.06 ± 6.14^{3}	79.90 ± 5.09^{2}	$86.53 \pm 4.31^{2,3}$

Comparisons are valid for each column. Different superscript letters and numbers indicate statistically significant difference ($p < 0.05$). *MF* Enamel Plus HRi Flow (UD3), *MH* Enamel Plus HRi(UD3), *NX3L* NX3 light-cure (Yellow), *NX3D* NX3 dual-cure (Yellow), *RXL* RelyX Veneer (A3), *RXU* RelyX Ultimate (A3)

DC in both groups was recorded by NX3D (P1: 52.24 ± 0.80; P2: 52.97 ± 1.32). On day 2, MF (78.50 ± 1.01), MH (79.68 ± 1.98) and NX3L (76.76 ± 4.66) had the highest DC in P1, while MF (78.50 ± 1.01) and MH (79.68 ± 1.98) had the highest DC in P2. Conversely, NX3D (58.73 ± 1.29) had the lowest DC in P1, while NX3D (58.19 ± 1.25) and RXU (62.23 ± 5.27) in P2. On day 7, MF, MH, NX3L, RXL showed the highest DC with both the protocols. On day 14, MF, MH, NX3L and RXL had the highest DC in P1 and P2. On day 28, MF, MH, NX3L and RXL had the highest DC in P1 and MH, NX3L and RXL in P2. On days 1, 2, 7, 14 and 28, the effects of P1 and P2 were not statistically different ($p < 0.05$).

Discussion

The longevity of an indirect restoration is directly affected by the resin luting agent [14, 15], and by its DC, whose evaluation may be performed by spectroscopic analysis [12, 16]. In particular, the DC of resin luting agents may influence the chemical and mechanical properties of these materials [17, 18]. Moreover, the DC is a critical factor for biocompatibility and colour stability and it is material dependent [4]: a high DC is essential for long-term functionality, while an inadequate DC can be detrimental to the success of dental restorations [2, 19–21]. In general, the maximum DC reached by resin cements is around 60% and increases after time [22].

In the present study, the kinetic of polymerization of various resin luting agents submitted to different curing protocols was evaluated in the first 5 min and, as further extent, the DC was studied over 28 days to better understand the chemical effects of the tested protocols. A 2.0 mm thick composite disk was used for simulating an indirect restoration. The obtained results showed that ~ 50% of the polymerization reaction of light curing materials occurred during the first 5 min, with the flow resin composite MF and the pre-heated high viscosity resin composite MH showing higher DC values with respect to the other light and dual resin cements. This fact could partly be explained by the higher percentage of filler load of MF and MH (77% and 80%, respectively), and consequently the lower matrix content, with respect to the other tested resin cements. During the polymerization process, the resin cement can create a so-called "uncured chamber": the monomers start to cure, and the material becomes rigid, trapping the unreacted monomers in the matrix. For this reason, the curing process takes 1–7 days to be completed [23, 24]. As in MF and MH, a low percentage of matrix content, and hence of monomer, could allow a thin layer matrix between monomers and fillers, decreasing the possibility to create uncured chamber. Our results are in agreement with Barceleiro et al. which suggested flowable resin composites are suitable alternative luting agents, when used below a thickness of 2.0 mm or less [25].

In summary, in the first 2 days, MF, MH and NX3L showed significantly higher DC compared with the other materials. After 7 and 14 days, DC of RXL resulted similar to that of MF, MH and NX3L, without significant difference, and the DC of dual-cure resin cements were significantly higher than that of light-cure resin cements. Although the dual cement is chemically activated, the low contribution of light curing is not enough to reach a high DC, thus requiring more time to complete the polymerization process and reach a high value. Another possible explanation could be the suboptimal concentration of curing inhibitors [26, 27]. Inhibitors can be added to resin cement to increase the material



manipulation and clinical working time [28]. Although the light curing materials also contain inhibitors, the concentration is relatively lower than the dual-cured materials. Consequently, a good balance between initiators and inhibitors is essential for clinical uses [28].

Our results agree with another study evaluating DC of resin luting agents when used under ceramic materials instead of composite. Filho et al. found that resin cements present low DC when the materials are dually activated through 2.0 mm of reinforced ceramic materials with translucency equal to or less than that of IPS Empress [5]. The translucency of IPS Empress was 10.37 with 2.3 mm of thickness [29]. In our study, the translucency of the composite disk used during the polymerisation phases was 10.07 and then it is less translucent than IPS Empress. Moreover, the translucency of our composite disk is lower than IPS e.max Press, IPS e.max CAD and Zirconia materials at different thickness [30]. Therefore, our result could relate to indirect restorative materials with a translucency equal or higher than 10.

Regarding the long-term evaluations, the tested protocols were not statistically different. Although different curing modes have been described in the literature, no data exists about DC effect of such "step luting" protocol P2, with a 28-day long evaluation. The P2 can be considered a modify pulse-delay curing, where the polymerization is initiated by a short flash of light followed by a waiting time of several minutes before the final cure is performed. However, in our P2, only 20 s were between the pre-curing phase (5 s) and the final curing phase (40 s).

In the tested P1, the total irradiance was 58,800 mW/cm^2 (1470 mW/cm^2 times 40 s) and no pre-curing phase was applied. In the tested P2 the total irradiance was 7350 mW/cm^2 for the pre-cure phase (1470 mW/cm^2 times 5 s), and 58,800 mW/cm^2 for the final curing (1470 mW/cm^2 times 40 s). No difference was noted between the tested curing protocols of P1 and P2 at 1, 2, 7, 14, 28 days.

In the P2 protocol, the low energy (7350 mW/cm^2) of the first 5 s of curing could initiate the conversion of the resin luting agent to a semi-solid state and could allow the indirect restoration to be fixed to the tooth, not affecting the chemical stability of the material. This finding is in partial agreement with Asmussen et al., although they used different pre-cured phases of 10, 20 and 40 s followed by 20 s of final curing, the final DC was not influenced by the low energy density of the pre-cure phase (from 250–16000 mW/cm^2) which is in accordance with our precured energy density of P2 (7350 mW/cm^2) [31]. However, in their study the final curing phase was immediately after the pre-curing phase, without the 20 s waiting time. At a high energy density of the pre-curing phase, the polymerization would proceed at a normal and high rate. While a pre-curing phase at low energy density could start the polymerization process by the formation of limited oligomers, building up discontinuous foci of polymerized material and creating microgel regions [32, 33]. This kind of microgel state would allow the clinician to easily remove the excesses when the material is starting to become hard.

Despite the lack of physical and evaluations about adhesion, we can conclude that the light curing resin cements achieved a clinically acceptable DC after 5 min. Furthermore, all the resin luting agents reached more than 50% DC after 1 day. Over the period, the light-cure luting cements had the higher DC values than the dual cured ones. In conclusion, we can reject the first null hypothesis, because our results suggest that all the tested materials did not reach the same DC, and accept the second one because of the two different curing protocols seem to not influence DC values, also over a long time. Then, the clinician can safely use the tested "step luting" protocol (5 s + 40 s) to lute the indirect restoration, simplifying the removal of cement excesses, in particular in the interdental space.

Funding Open access funding provided by Università Politecnica delle Marche within the CRUICARE Agreement.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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4. Conclusions

Extensive research and intense laboratory life made me touch the extraordinary world of nanotechnology, thus appreciating its interesting and limitless application in every field of dentistry. Indeed, there is a solid knowledge on nanotechnology and its discoveries, which daily surround our dental practice, being in continuous progression and certainly contributing to relevant benefits for both the clinician and the patient. The main topics addressed in the present dissertation represent only a small part of the extraordinary potential that nanotechnological revolution can bring.

Preventive dentistry is pervaded by countless innovations by exploiting the advantageous properties of biomaterials to safeguard the integrity of the enamel, promoting its remineralization. Moreover, several new devices can help dental clinicians to detect early diagnosis of oral potential malignant lesions.

In addition, several new devices overcame the challenge of aesthetic dentistry, based on original softwares that help clinicians to easily and exactly detect the color of the tooth to replicate it in the dental restoration. Then, the patient's desire to obtain a pleasant and aesthetic smile can be successfully satisfied, without worrying about concerns related to the possible side effects of dental whitening, thanks to the employment of nano-HA.

Nanotechnological applications in restorative dentistry assure to enhance the mechanical and physiological functions of the tooth restoration via novel nano-fillers and modern composites that provide an enhanced quality for direct and indirect restorations. Moreover, there is a continuously growing capacity for improvement and further advances in dental materials. A competitive and high quality of dental materials might be created if nanotechnology would be used, having in mind that other areas of its application could be explored in operative dentistry.



New frontiers are now opened thanks to the use of nanotechnology in dental tissues regeneration, by means of molecular biology, stem cells and tissue engineering approaches. Nanotechnology can provide not only innovative instruments and highly performing materials, but also offer new simplified strategies and techniques to obtain the best solution for every clinical situation, thus assuring the maximum patient's benefit using the best quality of therapies. The future of nanodentistry is obviously bright and it is becoming one of our most important tools for prevention, diagnosis and treatment.

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