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Po-Chun Chang
Kajorn Kungsadalpipob
I-Ping Lin
Huei-Jin Yin

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Acknowledgements

The 14th International meeting of the Asian Pacific Society of Periodontology (APSP) was successfully held in Bangkok, Thailand on August 29-30, 2022. Over 451 delegates from 18 countries (Australia, China, Hong Kong SAR China, India, Indonesia, Japan, Korea, Malaysia, Mongolia, Myanmar, Nepal, New Zealand, Philippines, Singapore, Taiwan, Thailand, United States, and Vietnam) attended this APSP meeting with the theme "Periodontal Innovations and Solutions for the 21th Century". The welcome address was given by Prof. Narongsak Laosrisin, Chairperson of the 14th APSP Meeting, and Dr. Suwannachai Wattanayingcharoenchai, Director–General, Department of Health, Thailand. The President's address was made by Prof. Ahmad Sharifuddin Mohd Asari. Welcome addresses were also made by Mr. Mutsuhisa Miyamoto, Chief Operation Officer Asia & Japan region, Sunstar Inc.; and Mr. Fumitomo Noritake, Director and Executive Officer, Lion Corporation.

The two-day program was very full, with 18 oral presentations, including 4 online presentations, delivered by speakers from 16 different countries. The keynote speakers were Professor Hom-Lay Wang (United States) and Professor Takanori Iwata (Japan). In addition, 81 posters were selected and scheduled for presentation.

The poster sessions were very successful. In keeping with the tradition from previous

meetings twelve prizes were awarded for the posters judged to be the best on the day in the categories of clinical research, laboratory research, clinical case reports and systematic/literature reviews. The abstracts of the winning posters are included in this volume.

This volume contains an impressive array of contributions from all around the Asian Pacific region and serves as a record of the invited presentations. Each of the chapters covers a unique aspect of current issues in periodontology as we understand them in 2022. As for past APSP Proceedings I am sure this volume will serve as a very important reference source in the years to come.

The APSP wishes to acknowledge the following sponsors for this meeting:

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On behalf of the APSP, I also want to acknowledge the tremendous support from Prof. Mark Bartold, the founder of the APSP Proceedings. The APSP Proceedings, the academic record of every meeting held by the APSP, has been published since 1995, and this tradition will definitely continue in the future events of the APSP.

Po-Chun Chang
APSP Editor
April 2023

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Biomimetic Materials: The Prospect of Periodontal Regeneration

Che-Chang Tu[1,2], *Po-Chun Chang*[1,2]

[1] Graduate Institute of Clinical Dentistry, School of Dentistry, College of Medicine, National Taiwan University, Taipei, Taiwan
[2] Department of Dentistry, National Taiwan University Hospital, Taipei, Taiwan

Introduction

Periodontitis is characterized by the combination of gingival inflammation with destruction of tooth-supporting structures, and frequently results in intrabony defects and furcation defects. The ultimate goal of periodontal treatment is to eliminate infection and to reduce periodontal pockets associated with periodontal regeneration, and to minimize soft tissue recession. Since the early 1970s, several surgical techniques—including bone replacement grafts (BRGs), guided tissue regeneration (GTR), the use of biologic agents, and the combination therapy—have been proposed, yielding a variety of clinical improvements (Cortellini and Tonetti 2000). To further increase the predictability and efficiency of periodontal regeneration, biomimetic regenerative materials have been widely developed and investigated over the last two decades.

The following sections briefly overview current available materials used in clinical periodontal regeneration procedures, and elucidate the progress of developing biomimetic materials in the field.

The current strategies of periodontal regeneration

Nonresorbable membranes for guided tissue regeneration (GTR)

Guided tissue regeneration (GTR) procedures involve placing a barrier membrane to prevent the downgrowth of junctional epithelium, preserving a space for the growth of bone and connective tissue (Caton *et al* 1987). In 1982, Nyman succeeded in forming new attachment in humans by using a cellulose acetate filters (Millipore®) barrier membrane (Nyman *et al* 1982). The first commercial barrier membrane was made of expanded polytetrafluoroethylene (ePTFE), an inert and biocompatible barrier permitting tissue integration, good for space maintenance. Clinical studies demonstrated that ePTFE membrane reduced periodontal pockets (PD) with clinical attachment gain, and facilitated bone fill in the intrabony and furcation defects for several years (McClain and Schallhorn 1993 ; Cortellini *et al* 1996 ; Gottlow *et al* 1992). The major drawbacks of using nonresorbable membranes include the need for a second surgery, highly technique-intensiveness, and a high incidence of membrane exposure rate compromised the outcome of regeneration (Nowzari *et al* 1996 ; Simion *et al* 1995 ; Page 1994).

Bioresorbable membranes for GTR

To avoid a second surgery for membrane removal, natural or synthetic bioresorbable membranes predominate the materials choice for GTR. Current evidence indicates that GTR procedures are equally effective when using bioresorbable and nonresorbable membranes (Cortellini and Tonetti 2000 ; Weltman *et al* 1997). There are three common types of bioresorbable membranes available on the market, including those composed of synthetic polyester-based polymers, collagen, and calcium sulfate (CaS). Type I and type III collagens from bovine or porcine are commonly used for bioresorbable membranes (Wessing *et al* 2018), and these membranes are resorbed by the enzymatic activity of macrophages and polymorphonuclear leukocytes in the human body (Tatakis *et al* 1999). The major disadvantage of collagen membranes is the lack of rigidity and their propensity to collapse (Bunyaratavej and Wang 2001). The synthetic polymers, including polylactic acid or poly(lactic-co-glycolic acid) (PLGA), degraded by hydrolysis in human body (Tatakis *et al* 1999), have also demonstrated regenerative capability in animals and humans, and thus feature in commercial barrier membranes for GTR (Wang *et al* 2019 ; Caffesse *et al* 1994 ; Sculean *et al* 1999). These polymer membranes also lack rigidity and stability, and their swift degradation might elicit a relatively strong inflammatory response, leading to resorption of the regenerated bone (Piattelli *et al* 1998 ; Hürzeler *et al* 1997). The resorption rate is mainly controlled by the extent of cross-linkage in the synthetic polymer and collagen membranes. CaS membranes are made by the hydration of

CaS-hemihydrate powder, producing a paste that can be molded and set into a rigid material. As such, CaS membranes are easily sterilized, inexpensive, completely resorbable, biocompatible, and moldable. The use of CaS membrane for GTR has been established by several clinical reports, with major disadvantages including their fast degradation and mild cytotoxic reactions (Sottosanti 1992).

In general, the bioabsorbable membranes are more tissue-compatible than nonresorbable membranes, and do not require a second surgical procedure. They have several common shortcomings, however, such as lower mechanical strength, and less space-making efficiency, so they are usually used in combination with bone replacement grafts to maintain membrane shape.

Bone replacement grafts (BRGs)

BRGs, mainly serving as scaffolds or fillers, were the first periodontal regenerative materials to be applied clinically. Grafts are categorized by their origins as follows: (1) autografts obtained from the same individual; (2) allografts obtained from a different individual of the same species; (3) xenografts obtained from a different species; and (4) alloplastic grafts made from synthetic and inorganic bone substitutes (Laurencin *et al* 2006).

Among BRGs, the autograft is the gold standard for bone regeneration according to its osteoinductive potential, with proven tissue regeneration success in periodontal defects (Hegedus 1923 ; Nabers and O'Leary 1964). However, autograft harvest needs a donor surgical site, which may result in additional surgical morbidity. To reduce complication, harvesting autograft intraorally, specifically at sites adjacent to defects, should be considered.

Allografts are often commercially available from tissue banks, and can be simply categorized into freeze-dried bone allograft (FDBA) and demineralized freeze-dried bone allograft (DFDBA). FDBA is frozen, defatted, and dehydrated bone; for DFDBA, the bone's inorganic portion is removed using hydrochloric acid to expose the osteoinductive molecules deposited in the bone matrix (Holtzclaw *et al* 2008). Hence, FDBA is considered an osteoconductive material, whereas DFDBA is considered osteoinductive (Urist and Strates 1971). Both raise the major concern of possible disease transfer from the donors. However, the risk of HIV infection has been calculated to be as low as 1 in 1 to 8 million (Mellonig *et al* 1992). Several clinical studies have proven the bone regeneration effect of FDBA and DFDBA in periodontal osseous defects (Bowers *et al* 1991 ; Mellonig *et al* 1976 ; Libin *et al* 1975). As DFDBA exhibits a higher osteogenic potential between these two (URIST and STRATES 1970), DFDBA may be preferred for periodontal regeneration.

Xenografts have long been used in periodontal therapy. Currently, the anorganic bovine-derived bone matrix (ABBM), from which the organic components of the bone have been removed, but the trabecular architecture and porosity retained, is commonly used both for treating periodontal defects and in implant surgery (Camelo *et al* 1998 ; MeIIonig 2000) ABBMs generally serve as osteoconductive scaffolds and are satisfactory for maintaining space.

Alloplasts are synthetic materials that contain some of the essential chemical components of natural bone (e.g., calcium and phosphate) but do not necessarily resemble bone's natural structure, and are made of different materials, most commonly calcium phosphate, including hydroxyapatite (HAp) and beta-tricalcium phosphate (β-TCP), bioactive glass, or polymers (Haugen *et al* 2019). Their major advantages are the standardized product quality and the absence of infectious disease risk. Given their weak regenerative ability, however, they are often applied with regenerative materials (Rosen *et al* 2000).

Bioactive molecules

There are three bioactive molecules available for periodontal regeneration: the enamel matrix derivative (EMD), platelet-derived growth factor-BB (PDGF-BB), and type-2 fibroblast growth factor (FGF-2).

EMD is purified from the enamel matrix of developing porcine teeth, and its major component is amelogenin (Zetterström *et al* 1997). Although EMD has been shown to benefit early wound healing, it remains difficult to draw broad conclusions owing to the limitations of current clinical studies (Miron *et al* 2016). EMD is effective and widely used for treating intrabony defects and furcation defects, and has shown comparable results as to the GTR procedure, with fewer complications (Miron *et al* 2016). EMD can be used alone but, in deep and non-contained defects, also combined with bone grafts for greater clinical attachment level gain and less gingival recession (Siciliano *et al* 2011).

PDGF was one of the earliest growth factors studied for its effect on wound healing. Multicenter clinical trials supported the effectiveness of 0.3 mg/mL of recombinant human PDGF-BB in combination with β-TCP to improve attachment level gain and bone gain in periodontal intrabony defects (Nevins *et al* 2005 ; Jayakumar *et al* 2011). The combination of rhPDGF with a β-TCP carrier is now commercially available, and the potential for regeneration of furcation defects still needs to be confirmed.

FGF-2 has potent angiogenesis-promoting action and also promotes the cellular proliferation of undifferentiated mesenchymal cells while retaining their pluripotency(Murakami 2011). Multicenter clinical trials support the effectiveness of FGF-2 to improve periodontal intrabony defects (Kitamura *et al* 2008 ; Kitamura *et al* 2011). The Ministry of Health, Labor and Welfare in Japan has approved 0.3% human recombinant FGF-2 for periodontal regeneration, and it has been commercially available since 2016.

Biomimetic materials

To overcome the obstacles of the clinical regeneration strategies, tissue engineering, which aims at creating functional tissues by properly coordinating cells, scaffolds, molecular signals, and sufficient blood supply, has been introduced as an alternative strategy for periodontal regeneration (Taba Jr *et al* 2005). Inspired by the hierarchical structure and the intricate microenvironment of native periodontium, biomaterial scaffolds have transitioned from application as a biologically passive structural frameworks to target-specific scaffolding systems that orchestrate periodontal regeneration by regulating stem cells and the local microenvironment (Jin *et al* 2019). Thus, developing biomaterials mimicking the structural complexity and the physiological functions of the periodontium has attracting a growing attention in the field.

Biomimetic scaffolds

Three-dimensional printing (3DP) technology has been applied to replicate the intricate microarchitecture of native structure, and achieve bespoke shapes to fit specific defects. and according to the slow degradation rate and moldability of the aliphatic polyester, 3D-printed polycaprolactone (PCL) and PLGA scaffolds have shown promising preclinical outcomes (Park *et al* 2012). However, the outcome of clinical periodontal regeneration by a 3DP PCL scaffold was not satisfactory, presumably due to the inferior cell affinity and bioactivity (Rasperini *et al* 2015 ; Asa'ad *et al* 2016). To closely mimic the 3D architecture of extracellular matrix (ECM) and to harmonize with the function and mechanical strength of native alveolar bone, natural polymers (including collagen and gelatin) combined with bioceramics (including HAp, β-TCP, and biphasic calcium phosphate) have become the most frequently used biomimetic materials for bone tissue engineering (Kuttappan *et al* 2016). Recently, a bio-ink containing 90% HAp and 10% PCL/PLGA was developed for fabricating 3DP hyperelastic biomimetic scaffolds which have shown favorable outcomes for regenerating large jawbone defects (Chang *et al* 2021). Bone regeneration was further robustified by infusing a RGD-functionalized alginate matrix with osteoid-like stiffness in the scaffold to mimic the biophysical microenvironment of ECM (Chang *et al* 2021).

While PDL is characterized by aligned collagen fibers to anchor the teeth, the biomimetic approach focuses mainly on developing fiber-guiding scaffolds. Decellularized tooth slices have been reported to support repopulation and differentiation of PDL cells (Son *et al* 2019). To properly control and produce the intricate micro-patterned PDL microarchitecture, polymeric 3DP scaffolds have been developed(Pilipchuk *et al* 2016). More recently, a 3DP waveform microfibrous collagen scaffold has been developed to provide a more bioactive cell–matrix interface capable of withstanding the functional loads on the PDL region (Lin *et al* 2021).

Bi- and multi-phasic scaffolds have been also introduced to accommodate the hierarchical soft and hard tissue compartment structure in the periodontium. By using a multi-scale computational design and transplanting genetically modified cells for the formation of human tooth dentin–ligament–bone complexes, Park et al. fabricated a composite hybrid (PDL–alveolar bone) polymeric construct and demonstrated well-oriented interfacial ligament fibers between the newly-formed cementum- and bone-like tissues *in vivo* (Park *et al* 2010). Lee et al. designed a multiphasic (cementum–PDL–alveolar bone) 3DP PCL/HAp scaffold integrating with region-specific biological signals and progenitor cells, and demonstrated the formation of distinct type of periodontal tissue within the microscaffold (Lee *et al* 2014). Huang et al. developed a biphasic cryogel scaffold by

constituting PDL-cementum using gelatin and alveolar bone, using gelatin/beta-tricalcium phosphate/HAp, and infusing region-specific biomolecules, and demonstrated the potential for reconstructing hierarchical periodontium in dogs (Huang *et al* 2020). Additionally, a construct composed of a 3DP biphasic (bone-PDL) PCL scaffold and stem cell sheets was invented by Vaquette et al., demonstrating good scaffold–tissue integration to achieve periodontal regeneration in sheep (Vaquette *et al* 2019).

Biomimetic membranes

Conventional barrier membranes offer only limited bioactivity and insufficient mechanical stability. The biomimetic membrane designs, in contrast, focus on providing zone-dependent bioactivity and/or mechanical properties. Such functionally graded membranes (FGMs) comprise functional layers on the surface to provide bioactivity, on an underlying core compartment to provide mechanical strength (Bottino *et al* 2012). The idea was first proposed by Bottino et al., who used sequential multilayer electrospinning (Bottino *et al* 2011). They used poly(DL-lactide-co-caprolactone) (PLCL) as the base material for the core compartment, and electrospun protein/polymer nanofibrous layers on the surfaces to mimic the ECM interface. On the inner surface, HAp was added to increase the membrane's rigidity, and, on the outer surface, metronidazole was added to control post-operative infection. By using the conventional collagen membrane as the core compartment, Ho et al. adhered electrospun drug/growth factor-loaded PDLLA nanofibers on membrane surfaces. Their original design incorporated antibiotics onto the outer surface to prevent infection, and PDGF on the inner surface to facilitate the repair and regeneration process (Ho *et al* 2017). To more efficiently control infection and the predictability of regeneration, they fabricated nanocomposites by encapsulating EMD-loaded nanospheres into the antibiotics-loaded nanofibers on the surfaces, and have shown preclinical effectiveness in regenerating alveolar ridge defects (Ho *et al* 2022).

Conclusion

Biomimetic approaches for periodontal and alveolar bone regeneration have been widely investigated in the last two decades, with various scaffold and/or membrane designs, using bioactive materials that mimic the biophysical environment and the native cell-matrix interaction, have shown favorable regeneration outcomes preclinically. However, evidence to prove the clinical effectiveness of. these approaches is still limited.

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Potential Applictation of mRNA-based Therapeutics for Periodontal Tissue and Peri-implant Bone Regeneration

Rangsini Mahanonda[1,2,3], Chantrakorn Champaiboon[1,2,3], Theeraphat Surisaeng[4], Noppadol Sa-Ard-Iam[2,3], Pimprapa Rerkyen[2,3], Wichaya Wisitrasameewong[1,2,3]

[1] Department of Periodontology, Chulalongkorn University, Bangkok 10330, Thailand.
[2] Center of Excellence in Periodontal Disease and Dental Implant, Chulalongkorn University, Bangkok 10330, Thailand.
[3] Immunology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand.
[4] Department of Conservative Dentistry, Faculty of Dentistry, Prince of Songkhla University, Songkhla 90110, Thailand.

Introduction

Currently, treatment of periodontitis and peri-implantitis to achieve complete restoration of damaged tissue and bone is frequently unmet and still challenging. The need for improvement in tissue regeneration strategy is rather urgent.

Recent advance in the field of "tissue engineering" for regenerative therapy has opened up possibilities with its multidisciplinary by nature combining concepts from cell biology, chemistry, wound healing, biomechanics, immunology, material and clinical sciences. Periodontal tissue regeneration and bone regeneration in edentulous ridge and around dental implants (ridge augmentation and peri-implant bone) have been actively studied (Larsson *et al* 2016).

Tissue engineering strategies

The current therapeutic strategies are based on the key concept of "tissue engineering" regarding the use of stem cells, scaffolds, and growth factors for regenerative medicine. Bioactive molecules or growth factors, in spite of their attractive properties as a tool to stimulate multi-potent cells within tissues to proliferate and differentiate into desired soft and hard tissues, their half-life of growth factors *in vivo* is relatively short, usually ranging from minutes to hours (Arakawa *et al* 2001). Consequently, supra-physiologic dose or multiple administrations are required (Mitchell *et al* 2016). Such high dose of growth factors may cause undesirable side effects and increase the therapy costs (Carragee *et al* 2011; D'Mello *et al* 2017). Inconclusive clinical efficacy of growth factors in periodontal and bone regeneration has been reported in various studies. So far, only recombinant human platelet derived-growth factor-BB (PDGF-BB) (GEM-21®, Osteohealth), bone morphogenetic protein-2 (BMP-2) (INFUSE®, Medtronic), and fibroblast growth factor-2 (FGF-2) (Regroth®, Kaken) are the few growth factors approved for clinical use as an adjunct to periodontal surgery in periodontal defects and dental implant ridge augmentation.

Instead of delivering growth factor proteins, gene therapy may provide better bioavailability of growth factor within the damaged tissue leading to greater tissue regeneration (Franceschi 2005). Earlier studies have initiated with plasmid DNA (*pDNA*) and viral vectors, as messenger RNA (mRNA) was thought to be unstable. *In vivo* gene transfer using fibroblast transfected with an adenovirus encoding BMP-7 (Ad-BMP-7) found to promote osteogenesis and cementogenesis in rat alveolar bone defect model (Jin *et al* 2003)ligament, and cementum is a major goal of therapy. Bone morphogenetic proteins (BMPs. *pDNA* encoding BMP-4 complexed with polyethylenimine (PEI) and encapsulated in poly(lactic-*co*-glycolic acid) scaffold demonstrated significant bone regeneration compared to a scaffold alone in rat cranial defect model (Huang *et al* 2005). The major challenge for using *pDNA* and viral vectors for clinical use of an oral disease, a non-life-threatening disease are safety concerns. Inserted DNA could integrate into the host genome**,** which could pose the risk of mutagenesis.

The key discoveries in stability and less innate immunogenicity properties of nucleoside-modified mRNA (Kariko *et al* 2005) and the efficient lipid nanoparticle (LNP) delivery system (Pardi *et al* 2015) have greatly contributed to the advancement of mRNA in the field of medicine.

mRNA technology: an emerging gene delivery system

For a few decades, due to the fragility of mRNA, it was abandoned as a gene therapy in favor of the more stable *pDNA* and viral vectors. The major limitations for using mRNA are due to its intrinsic immunogenicity and limited stability (Weissman and Karikó 2015). mRNAs bind to innate

sensing receptors; TLR3, TLR7, and the RIG-I like receptors, MDA5 and NOD2 (Goubau *et al* 2013). This binding leads NF-kB and interferon pathway activation, resulting in a pro-inflammatory cytokine response and inhibition of protein translation by type I interferon (Pollard *et al* 2013).

The immunogenicity of mRNA was overcome using nucleoside modification (Karikó *et al* 2005). Replacing uridine with pseudouridine into *in vitro*-transcribed (IVT) mRNA reduced mRNA-mediated immune activation and inflammation, while resulting in efficient protein translation (Karikó *et al* 2005; Karikó *et al* 2008). After several years of searching for the best nucleoside modification, the scientists found N1-methylpseudouridine-modified mRNA outperformed other modifications in terms of high translational capacity and reduced innate immune responses (Andries *et al* 2015). Removing the contaminated dsRNA generated during *in vitro* synthesis by high performance liquid chromatography further reduces immune activation and enhances protein translation (Karikó *et al* 2011).

The efficient delivery of mRNA is crucial for successful protein production *in vivo*. mRNA is sensitive to degradation by ribonucleases, therefore it must be made more resistant to degradation and more efficient for translation. Encapsulating RNA with lipids (Mintzer and Simanek 2009), polymers (Pack *et al* 2005), and peptides (Martin and Rice 2007) enhanced mRNA stability and also promoted cellular uptake and endosomal escape. The most studied RNA delivery system is the use of positively charged or cationic lipids, which has consistently shown promising results (Mintzer and Simanek 2009; Midoux and Pichon 2015; Pardi *et al* 2015). Cationic lipids are used to bind the negatively charged RNA, forming lipoplexes, which could protect mRNA from nucleases and facilitate endosomal escape, enhancing the efficacy of transfection and translation (Felgner and Ringold 1989; Guan and Rosenecker 2017; Kaczmarek *et al* 2017).

Lipid nanoparticles (LNPs) with neutral charge are currently recognized as the most promising delivery system for mRNA therapeutics and vaccines that allow high and sustained protein expression *in vivo* (Karikó *et al* 2012; Pardi *et al* 2015; DeRosa *et al* 2016). LNPs contain four lipid components; an ionized lipid, cholesterol, phospholipid (helper lipid), and lipid-anchored polyethylene glycol (PEG). Overall, producing large amounts of N1-methylpseudouridine-modified mRNA encapsulated with LNPs can be easily done in the laboratory without requiring a complex production and purification process.

mRNA as a new class of medicine:

After decades of research and development, mRNA has emerged as a safe and cost-effective technology platform for developing a new class of prophylactic vaccines and therapeutics. mRNA directs the body's cells to produce the encoded protein *in vivo*. Unlike DNA/viral vectors, mRNA is a non-infectious, non-integrating platform; thus, there is no potential risk of infection or insertional mutagenesis. mRNAs encapsulated with LNPs are internalized via endocytosis. Once inside the cells, the mRNA escapes from the endosome by an ill-defined mechanism and is then translated by a ribosome into protein that undergoes post-translational modification to form the mature protein (Guan and Rosenecker 2017). The destination of the encoded protein could be intracellular, integral membrane, or extracellular (Sahin *et al* 2014) (Figure 1).

Unlike proteins or peptides, the *in vitro* production of mRNA is simple, scalable, and does not require complicated cell culture and purification systems. Given the potential of an mRNA platform to encode any protein of interest and produce it directly *in vivo* (plug-and-play platform) mRNA vaccines and therapeutics are now of increased interest because they have been evaluated in many preclinical and clinical studies for preventing and treating a wide variety of diseases.

mRNA-based vaccine

Several years of research studies on mRNA vaccines culminated when the Chinese government reported a cluster of patients with pneumonia-like illness (later termed COVID-19, a disease caused by SARS-CoV-2) in late December 2019. mRNA vaccines designed to combat the COVID-19 pandemic have been rapidly developed 3 months after obtaining the viral genome sequence to clinical studies. Eleven months after the SARS-CoV-2 genome sequencing was published, COVID-19 mRNA vaccines with more than 90% efficacy were received emergency use authorization by FDA. This mRNA vaccine was the fastest vaccine ever developed and approved by FDA in history. So far, thousands of millions of mRNA vaccine doses have been administered globally and no serious side effects have been identified

(Baden *et al* 2021). Extremely rare cases of transient myocarditis and pericarditis have been observed following vaccination with COVID-19 mRNA vaccines (CDC and WHO 2021). The success of COVID-19 mRNA vaccines suggests that the future of mRNA vaccine technology looks extremely promising, especially for pandemic vaccines compared with conventional vaccine strategies.

mRNA-based therapeutics

In addition to prophylactic vaccines, mRNA-based therapeutics have also generated promising results. Administering erythropoietin mRNA encapsulated with LNPs led to substantial increases in serum erythropoietin levels in animals ranging from mice to pigs to nonhuman primates (NHPs) (Thess *et al* 2015). Delivering mRNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) protein through nebulization demonstrated a high production of functional CFTR in mouse and NHP lung epithelium (Robinson *et al* 2018). Based on these encouraging findings, mRNA encoding CFTR is now being clinically evaluated in humans. Interim results from a phase 1/2 clinical trial indicate that the CFTR mRNA was well tolerated and improved lung function (Translate Bio 2020). mRNA technology has also been used as an antibody therapy. Two studies described the use of mRNA-encoding neutralizing monoclonal antibodies against infectious diseases. Intravenous administration of mRNA encoding a broadly neutralizing monoclonal antibody (mAb) to human immunodeficiency virus (HIV)-1 encapsulated with LNPs resulted in large amounts of mAb in plasma 24 h post injection and protected humanized mice from HIV-1 challenge (Pardi *et al* 2017). mRNA encoding a potently neutralizing human monoclonal antibody encapsulated with LNPs (mRNA-1944) protected against chikungunya infection in NHPs (Kose *et al* 2019). Data from a clinical phase 1 study demonstrated that administering mRNA-1944 (0.1, 0.3, and 0.6 µg/kg) was safe and resulted in dose-related increases in antibody levels that exceeded the levels of antibody expected to be protective against chikungunya infection (> 1 µg/mL) following a single dose, and maintained antibody levels above protective levels for at least 16 weeks (Moderna 2020).

Regenerative medicine is also the area of intense investigation. The most advanced and exciting research in this area was the last year report of positive results from the phase 2 clinical trials for heart tissue regeneration by Moderna and AstraZenneca. During open heart surgery of eleven patients undergoing coronary artery bypass, VEGF mRNA were directly injected to their heart in order to grow new blood vessels and heart muscles at the infarcted area. The results met the primary end point of safety and tolerability (Collén *et al* 2022), a promising data of mRNA therapeutic for heart tissue regeneration. Now the phase 3 clinical trial is ongoing.

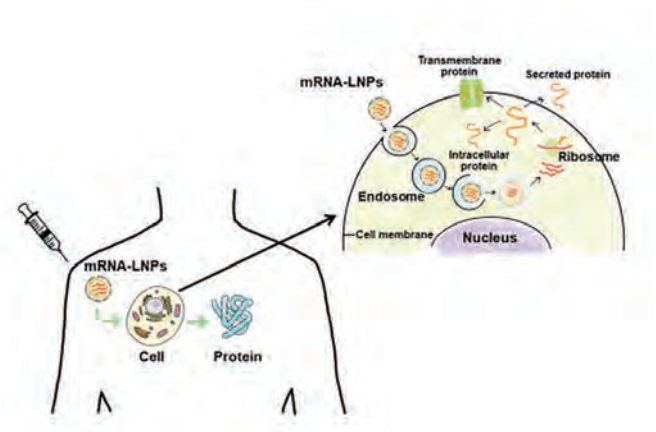

Figure 1. How mRNA technology platform works. mRNA in LNPs (mRNA-LNPs) delivered to the body by injection. Cellular uptake of mRNA-LNPs is via endocytosis. Once inside the cells, mRNA goes through the endolysosomal pathway, escape from the endosome to the cytosol and use ribosome for protein translation, whether being intracellular, transmembrane, or secreted protein (modified from Wisitrasameewong *et al* 2022).

Potential mRNA application for treatment of periodontitis and peri-implantitis

Challenges in treatment of severe periodontitis involve complex and multi-tissue integration (gingiva, periodontal ligament, cementum and supporting alveolar bone). Regarding dental implant therapy, alveolar ridge augmentation with bone and soft tissue is often required on edentulous site for dental implant placement in order to obtain functional stability and esthetic result. Similarly, challenges in treatment of peri-implant defects involve peri-implant bone regeneration for maintain peri-implant health and support. To overcome these challenges, we speculate that mRNA can be applied in multiplex, of which multiple mRNAs encoding growth factors for soft tissues and bone could be combined, formulated with LNPs in a single

administration and delivered into defects around teeth and dental implant (Figure 2). For example, mRNAs encoding PDGF-BB and FGF-2 may be suitable for soft tissue regeneration, whereas mRNAs encoding BMP-2 and BMP-9 could be optimal for bone regeneration. In addition, VEGF encoding mRNA could promote angiogenesis, an essential for all types of tissue engineering process (Wisitrasameewong *et al* 2022). This advantage of combined mRNAs-LNPs in a single delivery could allow us to customize design and construct a variety of mRNAs formulations that are suitable for tissue regeneration.

Scaffolds are important options that are required for structural support, cell homing, as well as sustained release of the mRNAs during tissue reconstruction (D'Mello *et al* 2017). Thus, the novel clinical application by the use of a specially designed 3D-printed scaffold loaded with either mRNAs-LNPs or mRNAs-LNPs-transfected stem/stromal cells may hold promise from the perspective of oral tissue regeneration, particularly in periodontal tissue and bone.

Conclusions

mRNA technology platform has been greatly boosted since the development of safe and effective COVID-19 mRNA vaccine. Recently, there have been more than 175 clinical trials opened using mRNA-based medicine. More than 50 clinical trials have been pending and ready to get started (https://clinicaltrials.gov/). It is agreeable that we have just entered a new era of medicine. In the field of mRNA-based regenerative therapy, the plug-and-play mRNA technology is simple and enables the design and production of any mRNA encoding growth factors encapsulated in lipid nanoparticles. We therefore speculate the delivery of the growth factor mRNA-LNPs with appropriate scaffold to the damaged soft tissue and bone around teeth and dental implant as valuable potential treatment for periodontitis and peri-implantitis. Future research and development of these new approaches are urgently required.

Acknowledgements

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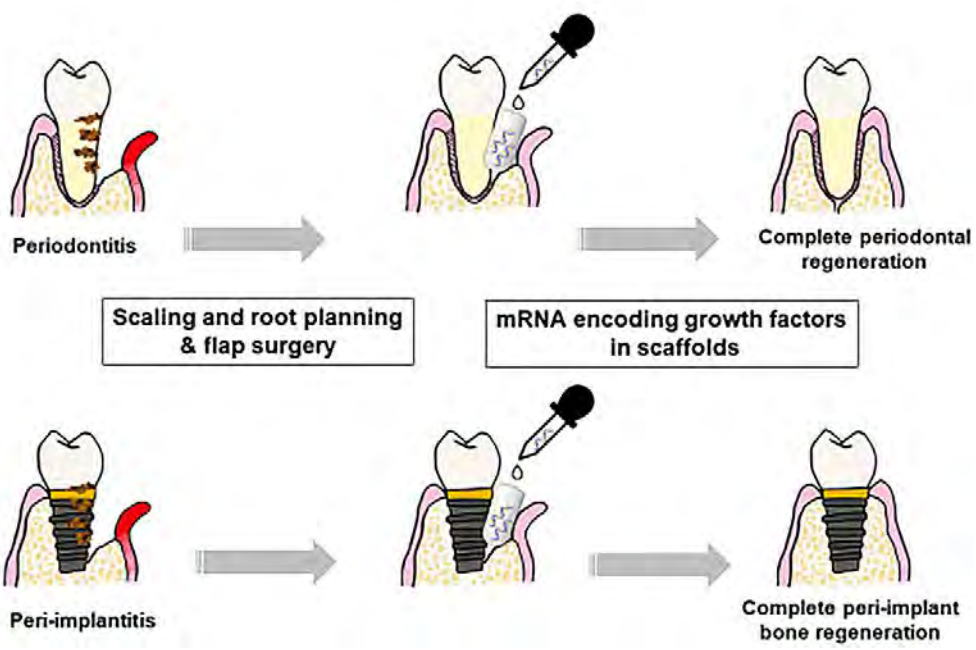

Figure 2. This diagram is our speculation how we can apply mRNA-based regenerative therapeutics for treatment of periodontitis and peri-implantitis. Growth factor-encoding mRNA(s) encapsulated in LNPs with scaffold could be delivered to the defects either in A) periodontitis or B) peri-implantitis (modified from Wisitrasameewong *et al* 2022).

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Inflammation-mediated Polymicrobial Emergence and Dysbiotic Exacerbation (IMPEDE) Hypothesis

PM Bartold
University of Adelaide, Adelaide, SA, Australia

Introduction

In 2020 a new new model for the pathogenesis of periodontitis termed "Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic Exacerbation" (IMPEDE") was proposed (Van Dyke, Bartold, & Reynolds, 2020). This was intended to assimilate with the 2017 World Workshop Classification of Periodontitis (Caton *et al*., 2018).

As early as 1981, Irwin Mandel observed that the specific flora of the deep pocket may be of significance only at a late stage of periodontal disease (Mandel, 1981). This elegantly surmises the basis of the IMPEDE model in that inflammation is probably a key event and the specific dysbiosis is a later "significant event". Indeed, periodontitis is now considered an inflammatory disease in which opportunistic and commensal bacteria, part of the normal oral microbiota, cause disease when the host is immunosuppressed. Thus, a concept of controlling periodontal inflammation to control periodontal infection has been proposed (Bartold & Van Dyke, 2017).

In 2018 a revised classification of the plaque-associated periodontal diseases was published based on the findings of the 2017 World Workshop Classification of Periodontitis (Caton *et al*., 2018). The plaque-associated periodontal diseases varied in severity that ranged from periodontal health through to advanced dentition-threatening disease. Accordingly, the periodontal diseases were classified by a sequential process of stage and grade allocation. Despite this new approach to disease classification, a number of questions remain unanswered:

- What is the process that leads to progression from the well-contained inflammatory response seen in gingivitis to the more widespread destruction seen in periodontitis?
- What drives the subgingival microbiome to become dysbiotic?
- Does dysbiosis arise spontaneously or is it a result of the ever-changing local milieu of the periodontal pockets?
- How does the dysbiotic microbiota interact with host innate and acquired immune responses?
- Is infiltration of periodontal tissues by bacteria causative, or a result of disease?

In this paper the interrelationship between periodontal inflammation and microbial dysbiosis is considered. This is crucial to understanding the initiating events associated with the development of periodontitis.

The Paradox of Periodontitis and Effectiveness of Oral Hygiene

A long-time dilemma in periodontology has been the so-called "Paradox of Periodontitis". Our clinics are full of patients who appear to have minor plaque deposits yet manifest with very significant periodontal destruction while others have significant deposits of plaque yet show little overt disease. These observations highlight the fact that bacteria are necessary but not sufficient for development of periodontitis. Accordingly, it seems appropriate to re-evaluate the role of oral hygiene & specific bacteria in the development of periodontitis.

Over 23 years ago it was noted that notwithstanding the awareness by patients for appropriate oral hygiene, sustained encouragement by the dental profession for better oral hygiene, and the resultant overall improvement in population oral hygiene habits, the prevalence of advanced periodontitis had remained largely unchanged (Bartold, *et al.*, 1998). The situation today is no different whereby 90% of all humans have gingivitis, mild, moderate and severe periodontitis affect 50% of all adults; and 2% of humans have severe periodontitis (McKenzie, 2022). Indeed, there is insufficient evidence to conclude that the prevalence of periodontitis has changed over time (Frencken, *et al*., 2017)

This raises several questions. Why has our focus on oral hygiene not always been successful? Is it that we

forgot the patient as a whole, with both the mouth and body connected? Several authors over the years have made similar observations. Improved oral hygiene, practices, devices and products, have been shown to have a positive effect as manifested by a reduction in plaque scores and in the prevalence of gingivitis, but they have had little effect on periodontitis (Page, 2000). Interestingly, the prevalence of severe forms of periodontitis has remained stable, thus factors other than plaque alone must be involved (Demmer & Papapanou, 2010). This raises the question: have we misunderstood, plaque/specific bacteria and host in the continuum of disease prevention and manifestation?

Plaque and Periodontal Risk

It is interesting to note that in one important periodontal risk assessment tool (Lang & Tonetti, 2003), dental plaque and specific periodontal pathogens do not form part of the assessment algorithm. This does not diminish the role of plaque/bacteria in the pathogenesis of periodontitis. It is evident that full mouth assessment of the bacterial load must have a pivotal impact in the determination of the risk for disease recurrence. However, studies to date have been unable to identify what level of plaque accumulation is compatible with maintaining periodontal health. For example, many patients can tolerate plaque accumulations of 20-40%. Nonetheless, plaque control remains central to viable preventive and treatment strategies. Therefore, it is important that the full mouth plaque score must be related to the host response (inflammatory parameters) of the patient.

Plaque has been noted to account for only 20% of the risk for periodontitis (Grossi, *et al*., 1994). Therefore, one must question what role does the remaining 80% of risk factors play in this equation. Clearly, periodontitis is a multifactorial disease. Accordingly, predisposing and modifying factors must be accounted for in pre-treatment assessment and the contributions of all risk factors to disease pathogenesis must be recognized and taken into account when trying to unravel the pathobiology of periodontitis.

Pathology of Periodontitis

The critical biological events to understand any pathological process and its management are: (1) Initiation of diseases, (2) exacerbation of disease and (3) resolution of the disease.

Initiation of Periodontitis: In most people (but not all), if dental plaque adjacent to the gingival margin is not disrupted regularly gingivitis will ensue (Kinane & Bartold, 2007). Gingivitis has been considered the precursor of periodontitis. However, of particular interest is that not all individuals who develop gingivitis proceed to develop periodontitis. Identification of the factors responsible for the conversion of chronic gingivitis to periodontitis remains unclear (Page & Schroeder, 1976). This observation was again highlighted in a review revisiting the Page and Schroeder model (Hajishengallis & Korostoff, 2017). Here, the authors observed that there was a dichotomy between the homeostatic protective and destructive host responses and that this issue still had not been adequately resolved.

Exacerbation of Periodontitis: This is when gingivitis progresses to periodontitis. The development and advancement of periodontitis occurs with developing microbial dysbiosis this appears to be an association rather than a causal event. Investigations into the exacerbation of periodontitis have been limited to associations with various events, but solid evidence for cause and effect has been difficult to substantiate. With increasing inflammation there is a significant change in the subgingival environment and this appears to coincide with alterations in the constituents and amount of subgingival biofilm and an associated microbial dysbiosis. Interestingly the influence of inflammation on the development of dysbiosis has been noted in other polymicrobial/inflammatory diseases (Bartold & Van Dyke, 2017; Kamada, *et al*, 2013; Lupp, *et al*., 2007; Plotnikoff & Riley, 2014; Zeng, Inohara, & Nunez, 2017). Nonetheless, neither the host response nor the microbial biofilm are the sole drivers of disease initiation and progression. Indeed, the timing and sequence of how both of these components of periodontitis have not been well defined.

Resolution of Periodontitis: Resolution of periodontal inflammation is defined as restitution of tissue homeostasis and a return of a commensal plaque microbiome in equilibrium with the host. In periodontitis, spontaneous resolution of inflammation does not occur because of the increased bacterial diversity and mass associated with the ongoing tissue inflammation and changing pocket milieu. Importantly, the evolving dysbiosis of the subgingival microbiome contributes to ongoing inflammation that further exacerbated the periodontitis condition. This ongoing vicious cycle can only be reversed by reducing the bacterial load leading to a reduction in inflammation, prevention of further tissue destruction and modification of the dysbiotic biofilm. Several

animal studies of experimental periodontitis have shown that by targeting resolution of inflammation, periodontal bone resorption improves, bacterial mass decreases and dysbiosis is reversed (Hasturk, *et al*., 2007; Lee, *et al*., 2016). Recently, in a human clinical trial, it was shown that topical application of the pro-resolving lipid mediator, Lipoxin A4 (LXA4), reduced gingival inflammation and increased the systemic levels of pro-resolution molecules systemically (Lee, *et al*., 2016). Thus, this therapy has the potential to modulate the progression and damage caused by periodontitis.

A Paradigm Shift in Thinking

The proposal by Socransky, *et al*. in 1998 that complexes of bacteria were associated with the shift from periodontal health to disease was a seminal moment in periodontology (Socransky, *et al*, 1998). However, it was always recognized that these groupings were "associations" and not "causative" for disease. While most microbes that colonise the human body are compatible with health, some can transform from a commensal to a pathogenic relationship with the host through mechanisms that are not well understood (Avila, Ojcius, & Yilmaz, 2009; Scher & Abramson, 2011). Indeed, a significant issue is the temporal relationship between inflammation and disease because it is not clear which comes first, the immune response or the alteration in composition of the biofilm (Dongari-Bagtzoglou, 2008). Nonetheless, it has been noted that periodontitis arises from an exuberant inflammatory response to the resident microbiota and this is aggravated by the overgrowth of some disease-associated bacteria (Wade, 2013).

Hypotheses for Periodontitis

Many hypotheses have been proposed to try to explain the pathogenesis of periodontitis. One of the earlier proposals was the Specific Plaque Hypothesis (Loesche, 1976) that highlighted the importance of a small number of specific "pathogenic" species. Subsequently the Non-specific Plaque Hypothesis was proposed (Theilade, 1986) that focussed more on the overall mass of microbiota. A more plausible and interesting was the Ecological Plaque Hypothesis (Marsh, 2003) where it was proposed that "periodontal pathogens" appear as a result of the disease rather than cause it? Two recent proposals called the Keystone Pathogen Hypothesis (Hajishengallis, Darveau, & Curtis, 2012) and the Polymicrobial Synergy & Dysbiosis model (Lamont & Hajishengallis, 2015) suggest that certain microbes modulate the host response to impair immune surveillance and tip the balance from homeostasis to dysbiosis.

Most of the more recent proposals have focussed on the so-called "keystone" bacteria as being the principal drivers of periodontitis. Although this seems reasonable, the evidence to support it is not so clear-cut because, in health, although the periodontal microbiome exists in a symbiotic relationship the host, putative periodontal pathogens are present, but not pathogenic.

Overall, periodontitis is an interaction between the inflammatory response, dysbiotic subgingival plaque biofilm and associated tissue damage modified by factors such as smoking, systemic diseases and genetics. Importantly, Inflammation always precedes the presence of dysbiotic microbes. Thus, it seems reasonable to focus our attention towards controlling the inflammation to control the infection.

Evolving Concepts

The one pathogen/one disease paradigm of the 1970's was replaced with a concept embracing the emergence of specific periodontopathic profiles that are associated with a number of ecologic determinants and host factors (Colombo & Tanner, 2019). An alternative paradigm supporting the central role of inflammation, rather than specific microbiota, in the early pathogenesis of periodontitis has also been proposed (Bartold & Van Dyke, 2019). Thus, while the predominant concept in periodontology today is that dental biofilm/plaque is the etiologic factor of periodontitis, this could be questioned. We know that bacteria are essential but insufficient to cause disease. To successfully treat the disease, the pathogenesis (inflammatory/immune responses) must be interrupted. Therefore, the biofilm should not be the sole treatment focus, since the pathogenesis is inflammation. For decades we have been attacking the problem from the wrong aspect and perhaps targeting the inflammation to control the infection may be an answer.

Inflammation & Dysbiosis in Periodontitis – Key Facts

The relationship between the periodontal microbiome and the development of periodontitis is multifaceted and complicated. The tenet that specific periodontal pathogens initiate dysbiosis and disease can be questioned because there is scant evidence to support a causative role for any putative keystone pathogen with initiation of periodontitis

in humans (Bartold & Van Dyke, 2019). Furthermore, the "putative pathogens" or "pathobionts" that are associated with periodontitis are very minor components of the early-stage biofilm (Kirst *et al*., 2015). The later transformation to a dysbiotic microbiota appears to be a response to the altered pocket environment and associated inflammation (Hasturk, *et al*., 2007; Marsh, 1994). A dysbiotic periodontal microbiome is linked to the development of periodontitis; however, whether disease is initiated by dysbiosis or is a result of inflammation has not been specifically proven (Hajishengallis, *et al*., 2012; Lamont & Hajishengallis, 2015).

Thus, understanding the nexus between inflammation and dysbiosis is critical (Van Dyke et al., 2020). The timing and sequence of microbial changes to periodontal inflammation is central to the pathogenesis of periodontitis. Understanding the inter-relationships between bacteria and inflammation is emerging (Lee, *et al*., 2016; Sima, *et al*., 2016). Inflammation forecasted disease progression and increased presence of periodontal pathogens in periodontitis occurs after onset of periodontitis (Tanner, *et al*., 2007). Dysbiotic changes induced by inflammation are more complicated than mere overgrowth of particular bacteria. Changes in the local pocket milieu induce alterations in the physiology, pathogenicity, and expression of virulence factors by the dysbiotic microbiota (Lamont, Koo, & Hajishengallis, 2018). Commensals can become pathobionts when specific genetic or environmental conditions are altered in the host (Janket, *et al*., 2021). In progressive disease, release of disease modifying products is upregulated by both commensals and pathogens (Yost, *et al*., 2015). These observations confirm that the microbiome and related inflammatory response are connected through bi-directional balance in health and disease.

A Unifying Hypothesis - Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation (IMPEDE) Model

This model is designed to accommodate the current Classification of Periodontal Diseases. In this classification, periodontitis is viewed within a continuum from health to disease through 4 stages of severity and complexity as well as extent and distribution. (Figure 1)

Four different stages of bacterial transitions have been identified during the transition from health to late-stage periodontitis, These are driven by inflammation, pocket formation, and bacterial composition (Figure 2) . The Inflammation-Mediated Polymicrobial Emergence and Dysbiotic Exacerbation (IMPEDE) Model is further illustrated in (Figure 3).

Stage 0 - Periodontal Health - presence of neutrophils are present in the gingival connective tissue but there are no clinical signs of inflammation. This is considered to be a homeostatic state.

Stage I - Gingivitis – With accumulating commensal plaque bacteria clinical signs of Inflammation (redness, bleeding) become apparent.

Stage II Initial/early periodontitis. Polymicrobial diversity is invoked due to the ongoing inflammation and dysbiosis within the microbial community is triggered.

Stage III - Dysregulated inflammatory response and deeper pocket formation. The dysbiosis if exacerbated by inflammation-mediated changes to the local environment and this results in a selfsustained feedforward loop

Stage IV – Late-stage periodontitis There is further Inflammation-mediated dysbiosis al microbiota and this results in further opportunistic infection and unregulated tissue damage.

This model can be used as a template for integration of treatment strategies into each of the periodontal disease classification stages (Figure 4). The IMPEDE model demonstrates how inflammation drives each periodontitis classification stage and, accordingly, the clinical condition. Inflammation-mediated polymicrobial dysbiosis and tissue damage can be exacerbated and progressive if no treatment is provided. By focusing on this inflammation driven problem treatment should be aimed at focusing on resolution of inflammation and tissue repair/regeneration.

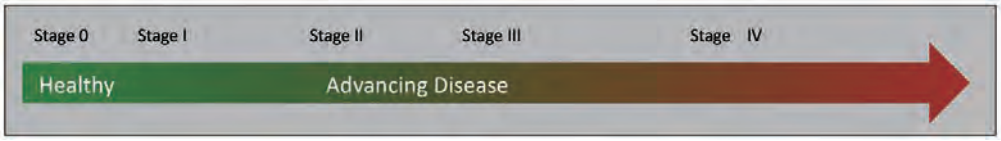

Figure 1. Plaque-associated Periodontal Disease Stages. Four different stages of periodontal disease have been identified during the transition from health to late-stage periodontitis

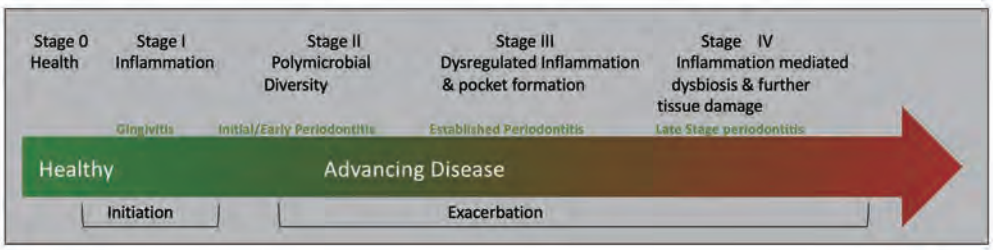

Figure 2. IMPEDE & Plaque-associated Periodontal Disease Stages.
Inflammation manifests within each classification stage as a principal driver of the clinical condition. Adapted from: Figure 2. Van Dyke TE, Bartold PM, Reynolds EC. The Nexus Between Periodontal Inflammation and Dysbiosis. *Front Immunol* 2020;11:9

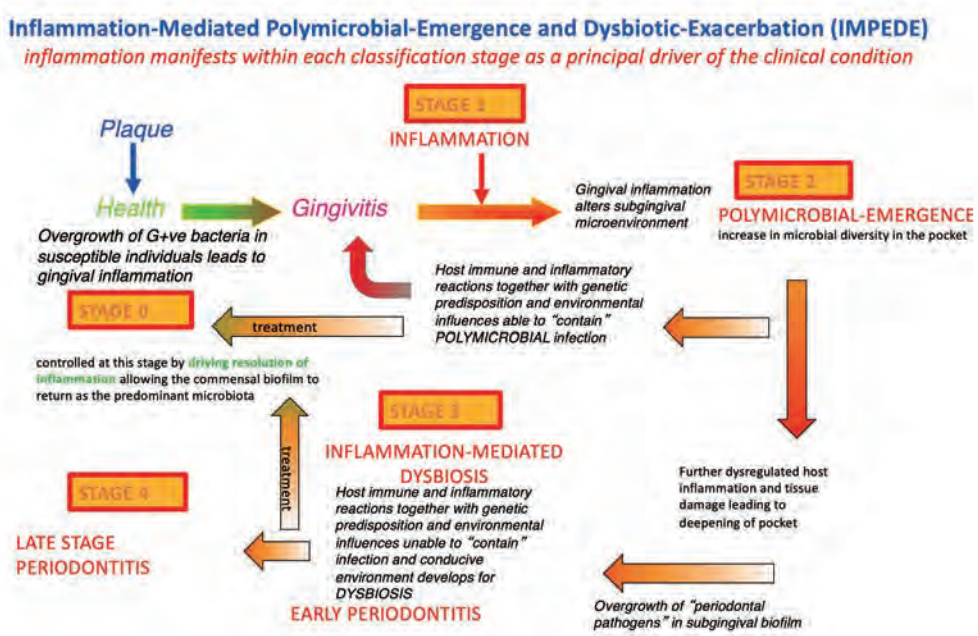

Figure 3. Inflammation-Mediated Polymicrobial Emergence and Dysbiotic Exacerbation (IMPEDE) Model. Inflammation is considered to be the main driver of plaque-associated periodontitis. This model demonstrates how periodontal health, gingivitis, and periodontitis may develop, be contained or progress through 5 stages (0-IV). Stage 0: periodontal health; Stage I: Gingivitis (inflammation); Stage II: Initiation/ early periodontitis (Polymicrobial diversity emerges); Stage III: Early-stage periodontitis: Inflammation-mediated dysbiosis and opportunistic infection and Stage IV: Late stage periodontitis. Adapted from: Figure 1. Van Dyke TE, Bartold PM, Reynolds EC. The Nexus Between Periodontal Inflammation and Dysbiosis. *Front Immunol* 2020;11:9

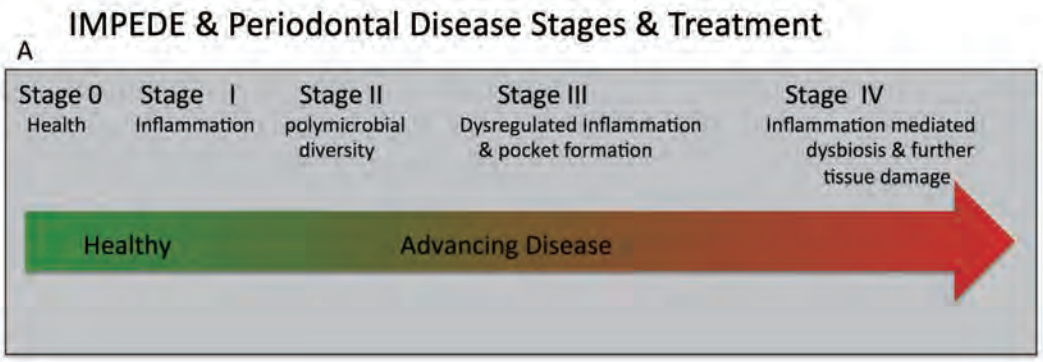

Figure 4. Inflammation-mediated polymicrobial dysbiosis and tissue damage can be exacerbated if no treatment is provided or can be driven toward resolution of inflammation and tissue repair/regeneration if treatment is provided. Adapted from: Figure 2. Van Dyke TE, Bartold PM, Reynolds EC. The Nexus Between Periodontal Inflammation and Dysbiosis. *Front Immunol* 2020;11:9

Conclusion

Contemporary periodontology is witnessing a merging of opposing concepts with the recognition of the crucial interplay between inflammation and microbiology in the pathogenesis of periodontitis. Emerging information indicates that the key driving force in the destructive features of periodontitis is ongoing and poorly controlled inflammatory host response. Only during the later stages of periodontitis does it become evident that specific microbial (dysbiotic) changes evolve and assume a role of considerable importance. It is important to note that this is not a novel proposal – just reworked to fit current findings and allow a logical fit with the 2017 classification.

Metagenomic, transcriptomic, proteomic, and metabolomics studies are providing key evidence to underpin earlier theories into believable working models. For example, changes in chemokine responses and microbial composition observed during experimental gingivitis demonstrate the variable host responses to a disruption in gingival homeostasis (Bamashmous *et al*., 2021). Consideration of such human variation in gingival inflammation may be useful to identify those individuals at high risk of developing periodontitis. Studies such as this underscore the plethora of host responses associated with differences in host immune outcomes and microbial biofilm evolution that may affect clinical manifestation of destructive inflammation.

The IMPEDE Model is consistent with, and complementary to, the 2017 Classification of Periodontal Diseases. This model allows us to see how resolution of inflammation has the potential to the microbial profile and restoration of microbiological and host homeostasis. Thus, the inter-relationship between periodontal inflammation and microbial dysbiosis can be utilized.

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PLAP-1/asporin is a Key Molecule in Association with Periodontal Disease and Obesity-Related Metabolic Disorders

Satoru Yamada

Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, Sendai, Miyagi, Japan

Introduction

Obesity is a major risk factor for type 2 diabetes and cardiovascular disease and is characterized by an excessive accumulation of adipose tissue. The expansion of adipose tissue is caused by proliferation and hypertrophy of adipocytes, which requires continuous remodeling of the extracellular matrix (ECM) to accommodate the expansion (Sun *et al* 2011). The flexibility of ECM enables healthy expansion of adipose tissue and the decreased flexibility in the adipose tissue ECM is often associated with metabolically unhealthy obesity (Smith *et al* 2019). It is accepted that adipose tissue fibrosis is a major contributor to obesity-associated metabolic dysfunction (Crewe *et al* 2017). Adipose tissue fibrosis may lead to apoptosis of adipocytes and chronic inflammation characterized by infiltrated macrophages (Thomas and Apovian 2017).

Although the deposition of fibrous collagen proteins such as collagen types I, III, and VI, is known to promote metabolic dysfunction in obesity (Datta *et al* 2018), the involvement of other ECM compartments, such as proteoglycans, is becoming increasingly evident (Pessentheiner *et al* 2020). The small leucine-rich proteoglycans (SLRPs) are the largest subfamily of proteoglycans and have been implicated in collagen fibrillogenesis (Kalamajski and Oldberg 2010). Among the class I members of SLRP, opposing roles of Biglycan and Decorin in obesity and meta-inflammation are shown in knockout mice models (Svärd *et al* 2019 ; Adapala *et al* 2012). However, the other member of class I SLRP, PLAP-1/asporin, has not been investigated. PLAP-1 (Periodontal Ligament-Associated Protein-1) was identified in our laboratory in human periodontal ligament (PDL) cDNA library(Yamada *et al* 2001 ; Yamada *et al* 2007) and is preferentially and highly expressed in PDL tissue(Henry *et al* 2001). Because PLAP-1 has a pro-inflammatory effect on periodontal tissue by suppressing pathophysiologic TLR signaling (Yamaba *et al* 2015), we determined whether PLAP-1 is also involved in adipose tissue biology.

Periodontal disease is a chronic inflammatory disease that progressively destroys periodontal apparatus including periodontal ligament and alveolar bone. Among the systemic diseases impacted by periodontal disease, obesity has been associated with an increased risk of periodontitis (Genco and Borgnakke 2013 ; Jepsen *et al* 2020). Consistent with epidemiologic evidence, high-fat diet (HFD) induced-obese mice have periodontitis (Blasco-Baque *et al* 2012). The investigation of association between obesity and periodontitis may help in understanding the mechanism of association and also improve knowledge of both the diseases.

To elucidate the function and the effect of PLAP-1 in adipose tissue, we first showed Plap-1 is expressed in adipose tissue and the expression is down-regulated in long-term HFD-fed mice. Plap-1 knockout (KO) mice were protective to weight gain, adipose hypertrophy, and metabolic disorders upon HFD feeding compared to control mice, possibly through less macrophage infiltration in adipose tissue. Furthermore, Plap-1 KO mice showed less HFD-induced alveolar bone resorption. In parallel with these in vivo analyses, in vitro experiments both by silencing Plap-1 and by treatment with recombinant PLAP-1 demonstrated that PLAP-1 promotes adipocyte differentiation of preadipocyte cell line, 3T3-L1 cells. The finding was further confirmed in the primary culture preadipocytes from Plap-1 KO mice. In addition, Plap-1 knockout mice showed different gene expressions of ECM in adipose tissue from wild-type (WT). Collectively, these data suggest that PLAP-1 has a protective role in high fat diet-induced metabolic disorder and alveolar bone loss by controlling adipose tissue expansion.

Materials and methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry. Mice with an HFD-induced

obesity were established based on a previously published method (Wang and Liao 2012). We fed 5-week-old WT and Plap-1 knockout (Plap-1 KO) mice, that were generated in our laboratory (Awata *et al* 2015), with 60 kcal % HFD (Research Diets, New Jersey, USA) or 10 kcal % normal chow diet (NC) (Research Diets) as the control diet. Food intake per cage per week was measured.

RNA extraction and quantitative polymerase chain reaction analysis

Maxilla, white adipose tissue (WAT), brown adipose tissue, brain, heart, lung, liver, stomach, small intestine, large intestine, pancreas, kidney, spleen, bone marrow, muscle, and gingiva were extracted from 8-week-old male WT mice. WAT was extracted from WT and Plap-1 KO mice after 16 weeks of feeding NC or HFD. Total RNA was extracted from these tissues and cultured cells using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) or PureLink RNA Mini Kit (Life Technologies, California, USA), respectively. Total RNA was reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, California, USA) and quantitative polymerase chain reaction (PCR) was performed with the StepOnePlus Real-time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix and gene-specific primers (S4 Table).

Glucose metabolic analysis

Before and after feeding NC or HFD, the glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed. For the GTT, mice were starved for 16 hours and were injected with 1.5 g kg-1 (bodyweight) glucose (Wako Pure Chemical Industries, Osaka, Japan) intraperitoneally. For the ITT, mice were injected with 0.75 U kg-1 (bodyweight) human insulin (Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally without starvation. Tail vein blood was collected at 0, 15, 30, 60, and 120 minutes after administration to measure blood glucose levels with ONE TOUCH Ultra (Johnson & Johnson Services, New Jersey, USA).

Biochemical serum marker test

After 16 hours of starvation, blood was collected from mice, incubated for 1 hour at room temperature, and then centrifuged (1,500 g, room temperature, 15 minutes) to obtain serum. Serum total cholesterol (T-CHO), triglyceride (TG), non-esterified fatty acids (NEFA), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and glucose concentrations were determined using L-type Wako CHO-H (Wako Pure Chemical Industries, Osaka, Japan), L-type Wako TG-H (Wako Pure Chemical Industries, Osaka, Japan), NEFA-SS Eiken (Eiken Chemical, Tochigi, Japan), Cholestest LDL (SEKISUI MEDICAL, Tokyo, Japan), Cholestest N HDL (SEKISUI MEDICAL), and Quick Auto Neo GLU-HK (Sino Test, Tokyo, Japan), respectively.

Quantitative analysis of alveolar bone resorption

Maxilla was collected from WT and Plap-1 KO mice before and after feeding NC or HFD and imaged by 3D micro X-ray CT R_mCT2 (Rigaku, Tokyo, Japan). These images were analyzed using TRI/3D-BON software (RATOC SYSTEM ENGINEERING, Tokyo, Japan). Distance between the alveolar bone crest and the cement-enamel junction in both the buccal and palatal side was measured at the distal root of the first molar and mesial and distal roots of the second molar in the apical direction using WinROOF software (Mitani Corporation, Fukui, Japan). The total value of these three distances was considered as the alveolar bone resorption.

Histological analysis

Epididymal adipose tissue was collected from WT and Plap-1 KO mice after 16 weeks of feeding HFD and then fixed in 4% Paraformaldehyde Phosphate Buffer Solution (Wako Pure Chemical Industries, Osaka, Japan) overnight. Samples were embedded in paraffin and sectioned at 3.0 μm with LEICA RM2245 (Leica Microsystems, Wetzlar, Germany). Sections were stained with Mayer's Hematoxylin (MUTO PURE CHEMICALS, Tokyo, Japan) and 1% Eosin Y Solution (Wako Pure Chemical Industries, Osaka, Japan). Stained sections were observed and imaged with ECLIPSE Ci (Nikon, Tokyo, Japan). The size of adipocytes was measured with ImageJ software.

3T3-L1 cell culture

3T3-L1 cells in Dulbecco's Modified Eagle's Medium (D-MEM; Life Technologies, California, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies,

California, USA) without antibiotics were seeded at 4 × 104 cells per well in a 24-well cell culture plate (Corning, New York, USA) and 2 × 105 cells per well in a 6-well cell culture plate (Corning, New York, USA), and then transfected with Silencer Select Plap-1 siRNA (Assay ID: s83722) or Silencer Select Negative Control siRNA (Life Technologies, California, USA) through Lipofectamine 3000 (Life Technologies, California, USA). Six hours after transfection, the medium was exchanged with D-MEM supplemented with 10% FBS and 60 μg/ml of kanamycin. Seventy-two hours after cell seeding, 3T3-L1 cells were incubated for 48 hours with adipocyte induction medium containing 0.5 mM isobutyl-methylxanthine (IBMX; Sigma-Aldrich, Missouri, USA), 1 μM dexamethasone (DEX; Sigma-Aldrich, Missouri, USA), and 10 μg/ml insulin (Sigma-Aldrich, Missouri, USA) to induce adipocyte differentiation, and then cultured with adipocyte maintenance medium containing 10 μg/ml insulin to maintain adipocyte differentiation for another 9 days (changed media every 2 days). Total RNA extraction and Oil Red O staining were performed with the cells seeded in 6- and 24-well cell culture plates, respectively.

Conditioned medium containing recombinant PLAP-1

3T3-L1 cells were infected with adenoviruses that carried LacZ or FLAG-tag mouse Plap-1 (Awata *et al* 2015), and the supernatant was collected as the conditioned medium (CM) after 48 hours. To confirm the expression of PLAP-1 in the CM, cultured cell supernatants (24 μl) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blotting. Horseradish peroxidase (HRP)-linked mouse anti-FLAG antibody (1:10,000; Sigma-Aldrich, Missouri, USA) was used for the detection of PLAP-1. Immunoreactive proteins were visualized by SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Illinois, USA) with the ImageQuant LAS4000 imager (GE Healthcare, New Jersey, USA).

Adipocyte differentiation utilizing PLAP-1 CM

3T3-L1 cells in D-MEM supplemented with 10% FBS, and 60 μg/ml of kanamycin were seeded at 2 × 104 cells per well and 8 × 104 cells per well in 24- and 6-well fibronectin-coated cell culture plates, respectively. Four days after seeding, confluent 3T3-L1 cells were cultured with adipocyte induction medium described above with or without 100% PLAP-1 CM for 48 hours, and then cultured with adipocyte maintenance medium with or without 100% PLAP-1 CM. Total RNA extraction and Oil Red O staining were performed with cells seeded in 6- and 24-well cell culture plates, respectively.

Isolation of primary preadipocytes and adipocyte differentiation

Primary preadipocyte isolation was performed according to a previously published protocol (Hayakawa *et al* 2018) with modifications. Minced adipose tissue from WT and Plap-1 KO mice was digested in D-MEM supplemented with 4,000 units/ml type II collagenase from Clostridium histolyticum (Sigma-Aldrich, Missouri, USA), 0.1 mg/ml DNase I (Roche, Basel, Switzerland), 10% FBS and 60 μg/ml of kanamycin at 37ºC for 30 min. The digest was filtrated with 40 μm Cell Strainer (BD Biosciences, California, USA), and centrifuged (500 g, room temperature, 10 minutes). The floating layer and cell pellet were obtained as mature adipocyte fraction (MAF) and stromal vascular fraction (SVF), respectively. SVF was suspended in D-MEM supplemented with 10% FBS and 60 μg/ml of kanamycin, and seeded in 24- and 6-well cell culture plates. On the next day, cells were rinsed three times with phosphate-buffered saline and cultured until reaching confluence. Cells were incubated with 10 μM pioglitazone (Sigma-Aldrich, Missouri, USA), 0.5 mM IBMX, 1 μM DEX, and 1 μM insulin for 48 hours to induce adipocyte differentiation, and then cultured with 100 nM insulin to maintain adipocyte differentiation. Total RNA extraction and Oil Red O staining were performed with the cells seeded in 6- and 24-well cell culture plates, respectively.

Oil Red O staining and quantitative lipid assay

Lipid accumulation in 3T3-L1 cells or preadipocytes isolated from mice was detected by Oil Red O staining utilizing Lipid Assay Kit (Cosmo Bio, Hokkaido, Japan). Cells were rinsed with phosphate-buffered saline and fixed in 10% Formaldehyde Solution (Wako Pure Chemical Industries) overnight. The fixed cells were washed with purified water and stained with Oil Red O solution for 15 min. After three washes, cells in purified water were imaged with ECLIPSE Ti (Nikon). For the quantification, isopropanol was applied to the stained cells, and absorbance at 540 nm of the extracted solution was measured with the Multiskan FC

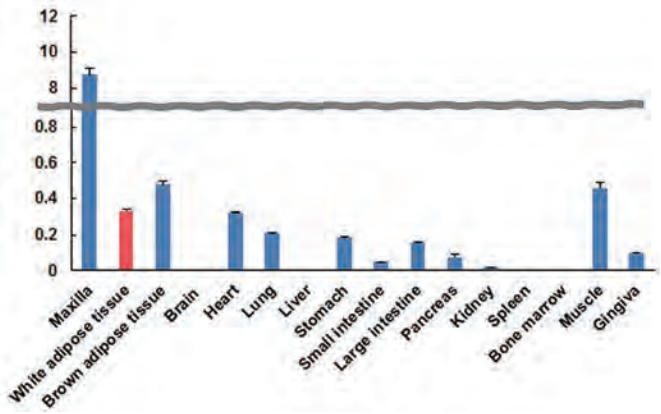

Figure1. PLAP-1 is expressed in adipose tissue.
Total RNA was extracted from various tissues and the expressions of Plap-1 were analyzed by real-time PCR analysis. Results show the mean ± SD of triplicate assays.

Microplate Photometer (Thermo Scientific, Illinois, USA).

Statistical analysis

Data were described using mean ± standard deviation. Statistical analyses were performed using the Student's t-test for paired comparisons and one-way analysis of variance for multiple comparisons with Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Plap-1 is expressed in adipose tissue and down-regulated in obesity

We have previously demonstrated that PLAP-1 is preferentially and highly expressed in PDL and has important roles in maintaining periodontal homeostasis by suppressing BMP-2 and TGF-β signaling. We analyzed Plap-1 expression in other tissues and organs in mice and found that Plap-1 was also expressed in WAT (Fig 1A). Thus, we hypothesized that PLAP-1 might have some crucial roles in WAT. To investigate the role of PLAP-1 in adipose and periodontal tissues, we fed mice with HFD and measured metabolic parameters.

Plap-1 deficiency ameliorates HFD-induced metabolic disorders

To study the roles of PLAP-1 under HFD condition, we fed WT and Plap-1 KO mice with HFD and measured their metabolic parameters. Plap-1 KO mice counteracted HFD-induced overweight (Fig 2A). There were no statistical differences of GTT and ITT between WT and Plap-1 KO mice when they were fed with NC. However, Plap-1 KO mice showed high glucose tolerance and enhanced insulin sensitivity after 16 weeks of HFD feeding (Fig 2B, 2C). Macrophage marker gene expressions are suppressed in adipose tissue of Plap-1 KO mice. We then analyzed adipokine expression in epididymal adipose tissue using real-time PCR (Fig. 2D). Adipoq, which exhibits a protective role in HFD-induced insulin resistance (Maeda *et al* 2002), was relatively highly expressed in Plap-1 KO mice but the expression did not reach statistical significance ($p = 0.148$), and there was no difference in the expression of Lep. It is well known that macrophages infiltrate into adipose tissue in HFD fed mice, and thus we also examined the gene expressions for macrophage markers. We found that Adgre1, which encodes F4/80, was expressed lower in Plap-1 KO mice although the difference did not reach statistical significance ($p = 0.064$). To characterize infiltrated macrophages in adipose tissue, we assessed expressions of M1 and M2 macrophage markers. M1 macrophage markers, Tnf and Ccl3, and an M2 macrophage marker, Clec7a (Ji *et al* 2012), were statistically lower in Plap-1 KO mice than in WT mice. These results suggest less macrophage infiltration in WAT of Plap-1 KO mice than the WT. We performed biochemical serum maker tests after 16 weeks of HFD feeding to investigate whether PLAP-1 has any influence on metabolic serum markers. Total cholesterol and glucose levels of Plap-1 KO mice were significantly lower than that of WT mice. On the other hand, the triglyceride level was higher than that of WT mice. These results suggest that Plap-1 KO mice may have a low lipoprotein lipase (LPL) activity, which decomposes triglyceride in the serum to free fatty acid and glycerol (Fig 2E). These data show that the deletion of Plap-1 improves metabolic disorder under HFD condition.

Plap-1 KO mice show less HFD-induced alveolar bone resorption

To investigate the involvement of PLAP-1 in periodontal health associated with HFD feeding, we assessed alveolar bone resorption of Plap-1 KO mice by μCT analysis (Fig 3). After 16 weeks of HFD feeding, alveolar bone resorption of Plap-1 KO was significantly less than WT mice (Fig 3), suggesting that Plap-1 KO mice were protective against HFD-induced alveolar bone resorption.

Endogenous PLAP-1 inhibits adipocyte differentiation in 3T3-L1 cells.

To further understand how PLAP-1 affects adipocyte differentiation, we utilized 3T3-L1 preadipocyte. We inhibited endogenous expression of Plap-1 in 3T3-L1 cells by siRNA, and induced adipocyte differentiation using these cells. Oil Red O staining showed significantly less lipid accumulation in Plap-1 knocked-down 3T3-L1 cells (Figs 4A and 4B). Furthermore, the real-time PCR analysis revealed that Adipoq and Fabp4 were significantly downregulated in Plap-1 knocked-down 3T3-L1 cells (Fig 4C). These results suggest that the endogenous PLAP-1 expressed by preadipocyte promotes its differentiation in a cell-autonomous manner.

PLAP-1 promotes adipocyte differentiation in 3T3-L1 cells

To investigate if exogenous PLAP-1 can promote adipocyte differentiation, we induced adipocyte differentiation of 3T3-L1 cells with the presence of recombinant PLAP-1. We induced adipocyte differentiation in the presence of PLAP-1 conditioned medium (PLAP-1 CM) obtained from PLAP-1-overexpressing 3T3-L1 cells. Oil Red O staining and quantitative lipid assay demonstrated that lipid accumulation in the presence of PLAP-1 was significantly enhanced compared to the control (Figs 5A and 5B). Furthermore, Adipoq and Fabp4 expressions were increased in the presence of recombinant PLAP-1 (Fig 5C). These results demonstrated that exogenous PLAP-1 could rigorously promote adipocyte differentiation.

Genetic depletion of Plap-1 inhibits adipocyte differentiation of preadipocytes isolated from mice

We isolated primary preadipocytes from subcutaneous adipose tissue of WT and Plap-1 KO mice, and induced adipocyte differentiation to investigate whether adipocyte differentiation in Plap-1 KO mice is also decreased in vivo. In accordance with the 3T3-L1 results, preadipocytes from Plap-1 KO mice showed significantly less lipid accumulation (Figs 6A and 6B). Furthermore, preadipocytes from Plap-1 KO mice showed low expressions of Adipoq and Fabp4 than WT cells (Fig 6C). These results indicate that adipocyte

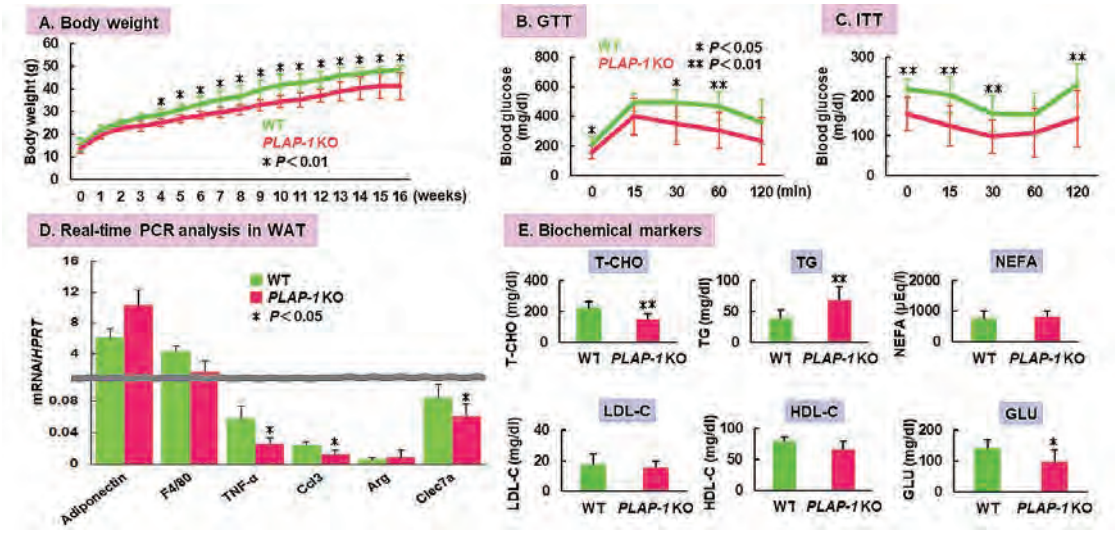

Figure2. Body weight and expansion of adipocytes in Plap-1 KO mice after HFD feeding.

(A) Body weight changes in WT and Plap-1 KO mice during HFD feeding. 5-week-old male WT and Plap-1 KO mice were fed with HFD and weighted weekly. WT (n = 7), Plap-1 KO (n = 9). Results show the mean ± SD. *: $p < 0.05$, **: $p < 0.01$ (B, C) GTT and ITT were performed in WT and Plap-1 KO mice after 16 weeks of HFD feeding. WT (n = 10), Plap-1 KO (n = 11). Results show the mean ± SD. *: $p < 0.05$, **: $p < 0.01$ (D) Real-time PCR analysis was performed in epididymal adipose tissue of WT and Plap-1 KO mice after 16 weeks of HFD feeding. WT (n = 4), Plap-1 KO (n = 5). Data are represented as relative expression to WT mice. Results show the mean ± SD of triplicate assays. *: $p < 0.05$ (E) Metabolic serum markers in WT and Plap-1 KO mice after 16 weeks HFD feeding. Serum levels of total cholesterol (T-CHO), triglyceride (TG), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), non-esterified fatty acid (NEFA), and glucose (GLU) were measured after HFD feeding. WT (n = 6), Plap-1 KO (n = 11). Results show the mean ± SD.

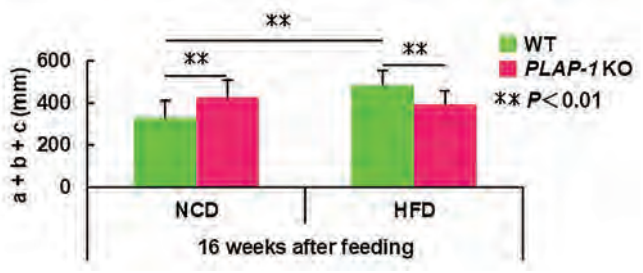

Figure3. Evaluation of HFD-induced alveolar bone resorption
Alveolar bone resorption was evaluated by μCT analysis in WT and Plap-1 KO mice fed with HFD, ×10 Distance between alveolar bone crest and cement-enamel junction was measured at distal root of first molar (a), mesial (b) and distal (c) root of second molar. Before feeding/WT (n = 10), before feeding/Plap-1 KO (n = 10), 16 weeks after feeding/NC/WT (n = 10), 16 weeks after feeding/NC/Plap-1 KO (n = 8), 16 weeks after feeding/HFD/WT (n = 10), 16 weeks after feeding/HFD/Plap-1 KO (n = 10). Results show the mean ± SD. *: $p < 0.05$, **: $p < 0.01$

differentiation was significantly suppressed in primary preadipocytes isolated from Plap-1 KO mice.

Plap-1 KO mice show different gene expressions of ECM in adipose tissue from WT

ECM surrounding adipocytes serves as a mechanical support and has a vital role in maintaining homeostasis in adipose tissue (Sun *et al* 2013 ; Khan *et al* 2009). It has been reported that the expansion of adipose tissue increased ECM gene expressions, and excessive ECM accumulation, especially of collagen I, III, and VI, caused fibrosis (Sun *et al* 2013). We sought the possibility that PLAP-1 affects the expression of other ECMs that have pivotal roles in adipose tissue fibrosis. Whole adipose tissue, SVF and MAF were obtained from WT and Plap-1 KO mice, and ECM gene expression was investigated. There was no difference in ECM gene expressions in whole adipose tissue of Plap-1 KO and WT mice (Fig 7A). However, the expression of

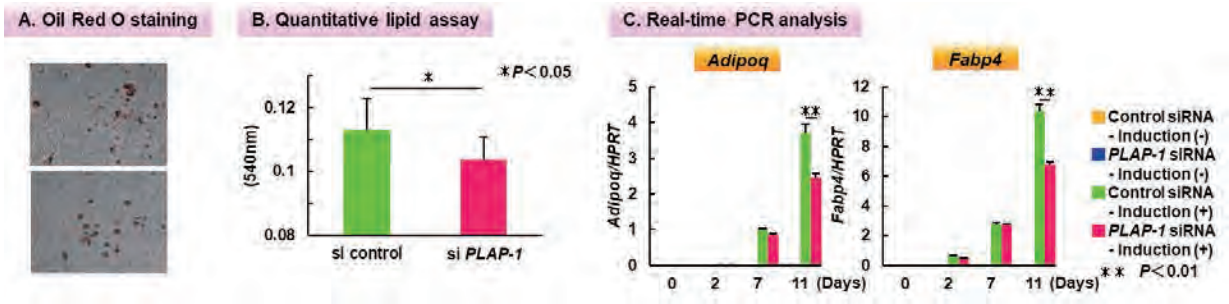

Figure4. Adipocyte differentiation of Plap-1 knock-down 3T3-L1 cells by siRNA
(A) Oil Red O staining of Plap-1 knock-down and control 3T3-L1 cells was conducted after adipocyte differentiation. (B) Quantitative lipid assay of Plap-1 knock-down and control 3T3-L1 cells was performed after Oil Red O staining (n = 4 in each group). Values represent the mean ± SD. (C) Real-time PCR analysis was performed to examine adipogenic gene expressions of Plap-1 knock-down 3T3-L1 cells during adipocyte differentiation. Results show the mean ± SD of triplicate assays. *: $p < 0.05$, **: $p < 0.01$

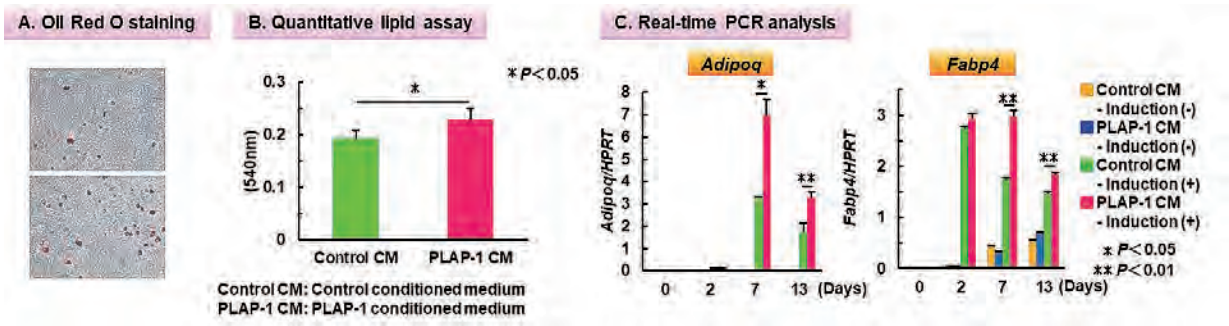

Figure5. AAdipocyte differentiation of 3T3-L1 cells in the presence of recombinant PLAP-1
(A) Oil Red O staining of 3T3-L1 cells in the presence of recombinant PLAP-1 was conducted after adipocyte differentiation. (B) Quantitative lipid assay of 3T3-L1 cells in the presence of recombinant PLAP-1 was performed after Oil Red O staining (n = 4 in each group). Values represent the mean ± SD. (C) Real-time PCR analysis was performed to examine adipogenic related gene expression of 3T3-L1 cells in the presence of recombinant PLAP-1 during adipocyte differentiation. Results show the mean ± SD of triplicate assays. *:$p < 0.05$, **: $p < 0.01$

Col1a1 Col6a1, Dcn, and Bgn in SVF of Plap-1 KO mice was significantly lower than those of WT. Furthermore, Col1a1 and Dcn expressions were higher, and that of Col6a1 and Bgn were lower in MAF of Plap-1 KO mice than WT (Fig 7B). These results demonstrated that ECM gene expression patterns in adipose tissue are different between WT and Plap-1 KO mice, and could be involved in the enhancement of fibrosis in adipose tissue.

Discussion

In this study, we investigated the HFD-induced metabolic change in Plap-1 KO mice and assessed the effect of PLAP-1 on adipocyte differentiation in preadipocytes. We demonstrated that PLAP-1 is expressed not only in PDL tissue but also in adipose tissue, and that it regulates the development of HFD-induced metabolic disorder and alveolar bone resorption. We also found that PLAP-1 promotes adipocyte differentiation in preadipocytes. Furthermore, we showed that PLAP-1 is involved in the expression of ECM, which has essential roles in the fibrosis of adipose tissue.

Obesity is one of the primary causes of diabetes, which is characterized by insulin resistance. It has been revealed that HFD causes insulin resistance, high concentrations of total cholesterol and glucose, and develops obesity in mice (Cha *et al* 2004 ; Jiang *et al* 2005 ; Fukuchi *et al* 2008). In our study, we established the obesity model mice by HFD feeding, and they exhibited insulin resistance and hyperglycemia. Adipocytes are surrounded by ECM, and the flexibility of ECM organized by collagen fibrillogenesis allows healthy expansion of adipose tissue. The rapid expansion of adipose tissue causes eventually leads to fibrosis due to excessive

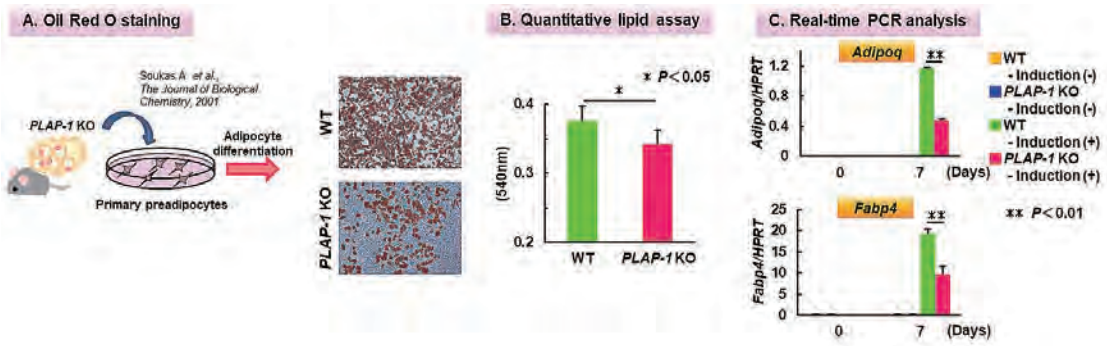

Figure6. Adipocyte differentiation of primary preadipocytes isolated from WT and Plap-1 KO mice

(A) Oil Red O staining of SVF isolated from WT and Plap-1 KO mice was conducted after adipocyte differentiation. (B) Quantitative lipid assay of SVF isolated from WT and Plap-1 KO mice were performed after Oil Red O staining (n = 4 in each group). Values represent the mean ± SD. (C) Real-time PCR analysis were performed to examine adipogenic related gene expression of SVF isolated from WT and Plap-1 KO mice during adipocyte differentiation. Results show the mean ± SD of triplicate assays. *: p < 0.05, **: p < 0.01

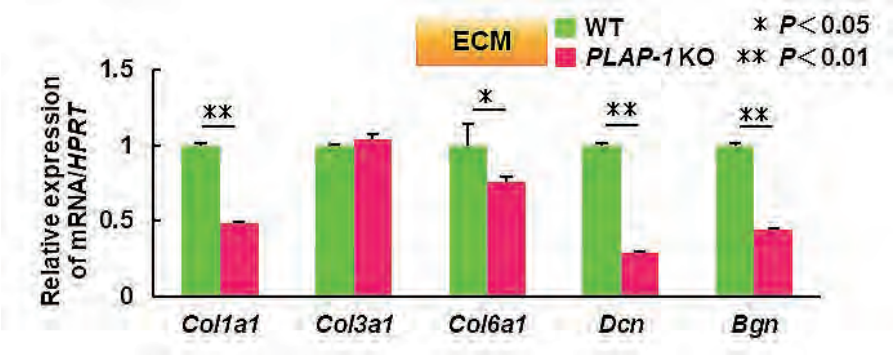

Figure7. ECM gene expressions in adipose tissue.

ECM gene expressions in adipose cells isolated from WT and Plap-1 KO mice were investigated by real-time PCR analysis. All data are represented as relative expression to WT. Col1a1: collagen, type I, alpha 1, Col3a1: collagen, type III, alpha 1, Col6a1: collagen, type VI, alpha 1, Dcn: Decorin, Bgn: Biglycan. Results show the mean ± SD of triplicate assays. *: p < 0.05, **: p < 0.01

expression of ECM. In our study, Plap-1 expression was significantly increased in adipose tissue of WT mice after eight weeks of HFD feeding. These results suggest that PLAP-1 may be induced and contribute to fibrotic changes in adipose tissue. Hypertrophy of adipocytes induced by HFD in Plap-1 KO mice was inhibited compared to WT mice, and one of the reasons for the inhibitory effect could be the influence on ECM flexibility via collagen fibrillogenesis.

We found that PLAP-1 cell-autonomously enhances adipocyte differentiation from preadipocytes to mature adipocytes in 3T3-L1 cells. PLAP-1 is known to bind TGF-β and suppress TGF-β-induced chondrogenesis (Kizawa *et al* 2005). Adipocyte differentiation is promoted by suppressing TGF-β expression in 3T3-L1 cells. It is suggested that TGF-β may inhibit adipocyte differentiation (Hirata *et al* 2012). Thus, PLAP-1 may promote adipocyte differentiation through suppression of TGF-β signaling.

In adipose tissue fibrosis, adipocytes surrounded by ECM undergo necrosis and M1 macrophages infiltrate the adipose tissue and cause inflammation(Cinti *et al* 2005). Macrophages produce inflammatory cytokines, such as TNF-α, and cause insulin resistance (Uysal *et al* 1997). In adipose tissue of Plap-1 KO mice, macrophage marker gene expression was lower compared to WT mice, suggesting that decreased recruitment of macrophages and decreased production of inflammatory cytokines may improve insulin resistance in adipose tissue of Plap-1 KO mice. PLAP-1 is expressed in cancer-associated fibroblasts of scirrhous gastric cancer and promotes invasion through activation of the CD44-Rac1 pathway (Satoyoshi *et al* 2015). PLAP-1 may promote the infiltration of macrophages mediated by CD44 activation. Interestingly, adipose tissue macrophage-derived Biglycan is recently reported to activate inflammatory genes in adipocytes (Han *et al* 2020). Further study is needed to determine whether macrophage secretes other SLRPs including PLAP-1.

In conclusion, we demonstrated that the absence of PLAP-1 inhibited HFD-induced metabolic disorder and alveolar bone resorption in vivo, and adipocyte differentiation with the change of other ECM expression in vitro. PLAP-1 may provide insights into the association between diabetes and periodontal disease.

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TWEAK, IL-6 and MMP-9: Novel Biomarkers linking stage III Grade B Periodontitis and Type 2 Diabetes Mellitus

Pradeep AR, Jayan A, Debnath K

Department of Periodontology, The Oxford Dental College, Bengaluru, Karnataka, India.

Introduction

Periodontitis is a polymicrobial dysbiotic disease resulting in the imbalance of the host defense mechanism, leading to immune-inflammatory response that can cause destruction of connective tissues and bone, which plays a key role in the progression of Periodontitis (Haffajee 1994). It results from the interaction between the biofilm and the host defense mechanisms which is sustained by various pro & anti-inflammatory mediators that may play as an antagonistic or synergistically to bring about various biologic activities (Tatakis and Kumar 2005). It is also regarded as a "silent disease" since patients have no or few symptoms for several decades which leads to disease progression and entails further impairment and impedes tooth preservation (Buset *et al* 2016).

Diabetes Mellitus (DM) is a complex metabolic disorder characterized by abnormal sugar, fat and protein metabolism with an increase in blood glucose level due to disruption in insulin production or defects in its action, which may lead to various systemic complications (Winer and Sowers 2004).

The tumor necrosis factor (TNF) and its receptors mediate their effects as a cytokine receptor subgroup that is known to attract considerable interest as a prospective source of therapeutic targets for the management of complex human disease (Winkles 2008). Chicheportiche (1997) first reported the discovery of tumor necrosis factor like weak inducer of apoptosis (TWEAK), which is a member of TNF-α family. It is considered a multifunctional cytokine that has pro-apoptotic, pro angiogenic and pro inflammatory effects (Sugito *et al* 2009). TWEAK induces various molecular mediators such as IL-6, MCP-9, IL8 and MMP-9 and the induction are augmented by interferon -γ (Kataria *et al* 2010).

Interleukin (IL-6) is pleiotropic cytokine. It exhibits both pro-inflammatory and anti-inflammatory roles. IL-6 are produced from the inflamed tissues of fibroblast, endothelial cells, dermal cells and mesangial cells. It is also known to be increased in inflamed periodontal tissues. The co-relation of IL-6 in peripheral blood and the amount of it produced in locally inflamed tissues appear to depend on the intensity of the disease (Takahashi *et al* 1994).

The extracellular matrix (ECM) provides the structural and biomechanical properties that are crucial for connective tissue functions. ECM interactions and signals transmitted via these receptors control many aspects of cellular life for proliferation, differentiation, maintenance and survival (Kumar MS 2006).

Matrix metalloproteinases (MMPs) are metal dependent endopeptidases which collectively can degrade most of ECM macromolecules (Birkedal-Hansen 1993). MMP-9 are secreted mainly by polymorphonuclear cells (PMNLS). MMP-9 especially degrade type IV collagen and is known to regulate basement membrane remodeling.

To date, no studies have been reported on TWEAK levels in crevicular fluid (CF) and serum and it has also not been co related with IL-6 and MMP-9 in stage III Grade B Periodontitis subjects with and without type 2 DM. It is a first of its clinical biochemical study designed to correlate the levels of TWEAK in CF and serum with IL-6 and MMP-9 in stage III Grade B Periodontitis subjects with and without type 2 DM.

Materials and methods

The study was conducted in the year 2020 from the month of August to November. It was conducted in full accordance to the Declaration of Helsinki 1975, revised in 2013. The research protocol was approved by the Institutional Ethical Committee and Review Board of The Oxford Dental College Bengaluru, India. Following the ethical clearance, the recruitment process was started. 42 subjects in the age group of 25-60 years were recruited from the outpatient section of Department of Periodontology, The Oxford Dental College, Bengaluru, India. Subjects who voluntarily accepted to be part of the study was made to sign a written informed

consent.

The subjects recruited had to satisfy the inclusion criteria set in the research protocol. It consisted of subjects in the 25–60-year age group, with at least 20 natural teeth, diagnosed with stage III grade B Periodontitis and had not received periodontal therapy within the preceding six months. Subjects having Body Mass Index (BMI) score in the range of 18.5-22.9 kg/m^2 and Waist Circumference (WC) <90 cm (men) and <80 cm (women) (WHO 2008) were selected for the conduct of the research. Well controlled type 2 DM subjects were recruited based on criteria given by American Diabetic Association 2018 and glycated hemoglobin levels.

Subjects with habits of smoking and alcohol consumption were excluded. Individuals suffering with rheumatoid arthritis, hypertension and other cardiovascular abnormalities, tumors, ulcerative colitis, Crohn's disease, renal disease or any other systemic disease that can alter the course of periodontal disease were also not recruited as per exclusion criteria. Pregnant and lactating females or those subjects who had received any medication like anti-inflammatory and antibiotics that can affect the periodontal status were also excluded.

Following the recruitment process, a full mouth periodontal probing along with interdental clinical attachment level charting was done for each subject and intra-oral peri-apical radiographs were taken using the long cone technique. BMI charting was done according to the WHO Guidelines (World Health Organization 2008) and diabetic status evaluated based on glycated hemoglobin levels (HbA1c) criteria of the American Diabetes Association 2018.

Subject Grouping

Subjects were selected randomly categorized into 3 groups based on gingival index (GI) (Loe & Silness), probing depth (PD), Clinical attachment level (CAL), radiographic evidence of bone loss and HbA1c levels. The group I (Healthy) consisted of 24 samples (12 CF and 12 serums) from 12 subjects with clinically healthy periodontium. Following assessing the gingival status using GI, the score obtained was 0, PD <3 mm and CAL=0 with no crestal bone loss as assessed from the radiograph. The group II (Stage III Grade B Periodontitis without type 2 DM) consisted of 30 samples (15 CF and 15 serums) from 15 subjects who had shown clinical signs of inflammation with GI score >1, PD≥5mm, CAL≥5mm with radiographic evidence of bone loss. The HbA1c values reported were < 6.5%. The group III (Stage III Grade B Periodontitis with well controlled type 2 DM) comprised of 30 samples (15 CF and 15 serums) from 15 subjects with clinical signs of gingival inflammation. The GI score recorded was≥1, PD ≥5mm, CAL≥5mm with radiographic evidence of bone loss. The HbA1c levels were <7%.

Site selection

The clinical examinations, intra-oral radiographic examinations, group allotment, sampling and site selection were carried out by one examiner. Following the detailed examinations and allocations of the subjects to respective groups; on the subsequent day samples were collected. This was done to prevent the contamination of CF with blood associated with probing of inflamed sites. The clinical assessment was performed with a University of North Carolina (UNC-15) periodontal probe (Hu- Friedy, Chicago, IL, USA). The collections of CF from test sites were based on the signs of inflammation, highest CAL and radiographic evidence of bone loss. In healthy subjects to obtain adequate fluid volume, pooling of the samples were obtained from multiple sites.

GCF collection

The subjects were made to sit comfortably in an upright position on the dental chair, after which the air drying and isolation with cotton rolls of the selected test sites were performed. Supragingival plaque was removed gently using a Universal Gracey curette #4R/4L to avoid contamination and blocking of micro capillary pipette. CF was collected by placing white color coded 1-5µl calibrated volumetric micro capillary pipettes obtained from Sigma- Aldrich Chemical Company, USA. A standardized volume of 3µl was collected in 10-20 minutes from each test site by placing the tip of the pipette extracrevicularly. After collection of CF, the samples were assigned to a particular group based on GI score, PD, CAL and radiographic evidence of bone loss. The samples contaminated with blood and saliva was discarded. The CF pooled from test and healthy sites were transferred to a sterile eppendrof vial containing 200µl of phosphate buffer saline (PBS) covered with a tin foil and stored at -70 °C until analyzed. Periodontal treatment (scaling and root planing)

were performed for group II and group III subjects at the same appointment following the CF collection.

Serum collection

Following disinfection of the skin over the antecubital fossa, 2ml of blood was withdrawn from by venipuncture using 20-gauge needles and immediately transferred to the laboratory. Blood samples were allowed to clot at room temperature and after one hour it was centrifuged at 2200-2500 rpm for 15 minutes. From this, the serum was extracted from blood and transferred into a vial and stored at -70 °C till the assay procedure.

TWEAK, IL -6, MMP-9 analysis

The samples were assayed for TWEAK, IL-6 and MMP-9 using their respective enzyme linked immunosorbant essay (ELISA) kits according to manufacturer's instructions. HUMAN TWEAK ELISA KIT was used for TWEAK and IL-6 used HUMAN IL-6 GENLISA™ obtained from (Krishgen Biosystems India) respectively. HUMAN MMP-9 ELISA kit was used for MMP-9 obtained from Kineisis Dx, USA. These assays employ a pre-coated monoclonal antibody specific for each TWEAK, IL 6 and MMP-9 on 96 well plate. Standards and samples were pipetted into the wells and any TWEAK, IL 6 and MMP-9 present was bound by Biotin labeled immobilized antibody and captured by biotinylated antihuman TWEAK, IL 6 and MMP-9 polyclonal antibody (as per the kit). Addition of HRP conjugated streptavidin was done. A substrate solution was added after washing. The colors developed in proportion to the bound biomarkers quantified and were monitored using a micro plate reader until an maximum optical density was reached. A stop solution of 50 µl was added and the optical density was read at 450 nm. The concentrations of each biomarker (TWEAK, IL-6 and MMP-9) were estimated based on the optical density values of the standards provided with the respective kits.

Statistical analysis

The data were analyzed using a statistical software program (SPSS Inc. Version 10.5, Chicago, IL, USA). At the initial stage of the study; power of the study was calculated. Based on the power of the study and confidence interval of 95% ($p<0.05$), sample size was determined. The correlation between CF and serum for each molecule and other clinical parameters was statistically significant or not was determined

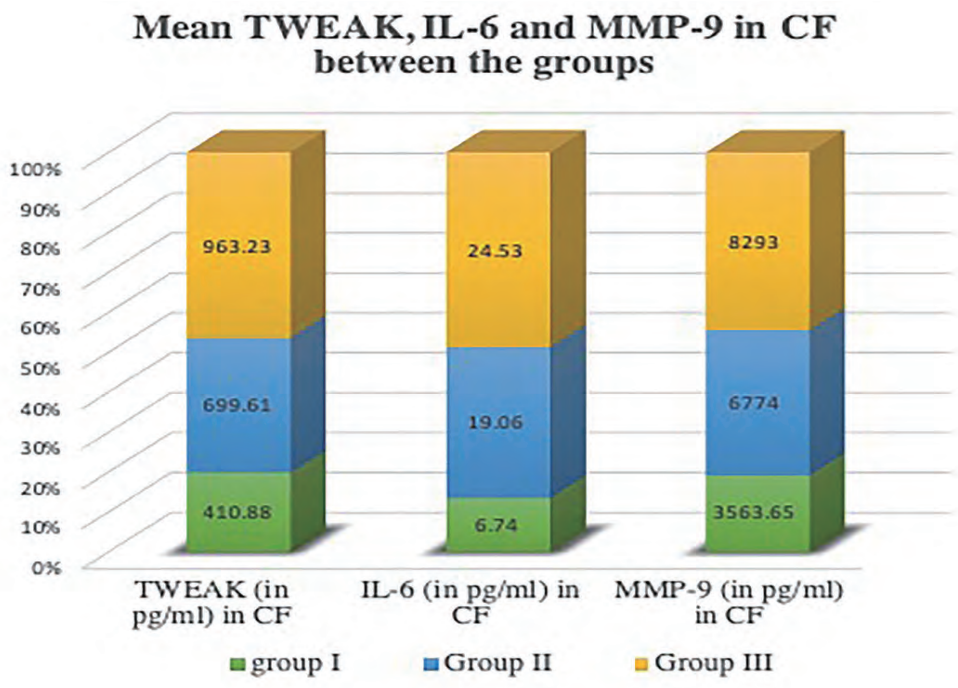

Figure 1.Mean distribution of TWEAK, IL-6 and MMP-9 in CF between group1, 2 and 3.
The result demonstrated an increase in the biomarkers in group 3 which was statistically significant with p value <0.01

by Spearman's correlation coefficient with P value <0.01.

Results

The descriptive statistics along with the mean CF and serum concentration for all groups are tabulated in Tables 1 for TWEAK, IL 6 and MMP-9. The mean TWEAK, IL 6 and MMP-9 concentrations in both CF and serum were highest for group III followed by group II and least concentration was observed in group I as illustrated graphically in figure 1 and 2.

The Spearman's correlation test was done to assess the biomarkers with each other in both CF and serum as depicted in table 2A, 2B and 2 C respectively. The analysis of the test showed that IL-6 levels in CF have a strong correlation for TWEAK, IL-6 and MMP-9 in serum whereas a moderate correlation was observed with CF TWEAK levels. The serum TWEAK values have shown a strong correlation with both serum and CF IL-6 and a moderate association with MMP -9 in CF and serum. The serum MMP-9 values have shown a strong correlation with both serum and CF IL-6 levels and strong correlation for serum and CF TWEAK levels. The correlation of CF and serum fluid levels of TWEAK, IL-6 and MMP-9 were statistically significant ($p<0.01$) in all the 3 groups.

Discussion

The present cross-sectional study assessed CF and serum levels of TWEAK, IL-6 and MMP-9 in periodontally healthy, stage III grade B Periodontitis and stage III grade B Periodontitis subjects with type 2 DM. The results indicated that the levels of TWEAK, IL-6 and MMP-9 increased in both CF and serum in subjects with stage III grade B Periodontitis with and without type 2 DM when compared to periodontally healthy subjects. The clinical parameters including PI, GI, PD and CAL have also shown a positive correlation with all the three markers which indicated that as the disease progress the levels also increases both locally and systemically. A dysregulated immune system is central to the pathogenesis of Diabetes and associated complications and Periodontitis is considered as the sixth complication of Diabetes (Taylor JJ and Preshaw PM 2013).

TWEAK and its receptor Fn14 leads to the activation of several pro-inflammatory mediators like IL-6, IL-8, MMP-9. It is also known to regulate immune responses and angiogenesis, tissue repair/ regeneration, apoptosis with an effect on osteoblast and osteoclast (Perper *et al* 2006). Periodontally affected tissues overexpressed TWEAK which induced endothelial activation molecules within human gingival fibroblast. Kataria NG (2009) found a moderate correlation between expression of TWEAK with inflammation and bone loss. Higher levels of TWEAK was expressed in periimplantitis group compared to the healthy group (Yakar *et al* 2019)

Leira Y and Ameijeria P (2020)(Leira *et al* 2020) have also evaluated an increased serum TWEAK levels in Periodontitis patients. TWEAK Fn14 monoclonal antibody revealed that a human macrophage cell line, THP1, expressed high basal level of TWEAK. This stimulation further induces the expression of MMP-9 and treatment of THP-1 cells with TWEAK induces IL-6 (Kim *et al* 2004).

IL-6 a cytokine is also known to show an increased levels in CF of Periodontitis subjects and is closely related to clinical severity of Periodontitis (Irwin and Myrillas 1998). Polymorphonuclear leukocyte derived MMPs (MMP-8, MMP-9) are the main proteases related to tissue destruction and remodeling events in periodontal diseases. Selective inhibitors of gelatinase and collagenase have shown to inhibit bone resorption in vitro (PA Hill 1995). Therefore, evaluation of these markers is equally important to understand the pathogenic mechanism and also to be used as therapeutic strategy.

CF levels of TNF α, IL-1β, MMP-8 and MMP-9 were reduced significantly following periodontal treatment in type 2 DM (Correa FOB 2010). Polak D and Shapira L (2018) (Polak and Shapira 2018) have observed an elevated level of IL-1β, IL-6 and RANKL/OPG ratios in patients with diabetes and Periodontitis as compared to Periodontitis patients. The results of the present study showed that serum concentrations of IL-6, MMP-9 and CF concentration of TWEAK were higher in group III which indicated that these markers were elevated in transition from health to an inflammatory state.

However, a study done by Acharya AB (2019)(Acharya *et al* 2019) had also found a negative correlation between TWEAK and insulin resistance as TWEAK levels can also be affected at an early phase of insulin resistance which was in contrary to the present study.

TWEAK expression in chronic inflammatory disease models were characterized by persistent TWEAK /Fn14

Table 1. Descriptive statistics of study population of TWEAK, IL-6 and MMP-9 (mean ± SD).

Study group	Group I (n=12)	Group II (n=15)	Group III (n=15)
Age (in years)	26±2.09	43.73±5.64	43.73±5.64
PI	0.98±0.22	1.36±0.59	1.51±0.42
GI	1.35±0.43	2.00±0.48	2.09±0.49
PD	2.10±0.63	4.43±1.95	6.01±0.44
CAL	0.00±0.00	4.3±1.20	5.58±0.70
BMI (kg/m2)	21.94±1.55	21.88±1.66	23.11±1.08
HbA1c	4.54±0.21	4.53±0.27	6.71±0.15
TWEAK (in pg/ml) in CF	410.88 ±118.90	699.61± 90.04	963.23±58.56
TWEAK (in pg/ml) in serum	402.34±132.41	622.50±115.30	676.49±70.33
IL-6 (in pg/ml) in CF	6.74±0.54	19.06±3.59	24.53 ± 4.35
IL-6 (in pg/ml) in Serum	6.44±0.84	22.92±4.24	26.24 ±3.13
MMP-9 (in pg/ml) in CF	3563.65± 411.52	6774±1797.77	8293± 5013.69
MMP-9 (in pg/ml) in serum	4692.32± 435.25	22595.09 ±1665.62	25007.39 ± 5938.63

Table 2A. Spearman's correlation test to assess the relationship between IL 6 with TWEAK and MMP-9 in CF and serum

IL-6 in CF										
Overall (Group I, II, III)	IL-6 in serum		TWEAK in CF		TWEAK in serum		MMP-9 in CF		MMP-9 in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.84	<0.001*	0.52	<0.001*	0.87	<0.001*	0.77	<0.001*	0.8	<0.001*
IL-6 in serum										
Overall (Group I, II, III)	IL-6 CF		TWEAK in CF		TWEAK in serum		MMP-9 in CF		MMP-9 in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.84	<0.001*	0.59	<0.001*	0.86	<0.001*	0.73	<0.001*	0.79	<0.001*

*Significant at p value <0.001

Table 2B. Spearman's correlation test to assess the relationship between TWEAK with IL 6 and MMP-9 in CF and serum

TWEAK in CF										
Overall (Group I, II, III)	TWEAK in serum		IL-6 in CF		IL-6_serum		MMP-9 in CF		MMP-9 in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.67	<0.001*	0.52	<0.001*	0.59	<0.001*	0.70	<0.001*	0.57	<0.001*
TWEAK in serum										
Overall (Group I, II, III)	IL-6 CF		TWEAK in CF		TWEAK in serum		MMP-9 in CF		MMP-9 in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.67	<0.001*	0.86	<0.001*	0.86	<0.001*	0.75	<0.001*	0.74	<0.001*

*Significant at p value <0.001

Table 2C. Spearman's correlation test to assess the relationship between MMP-9 with IL 6 and MMP-9 in CF and serum

MMP-9 in CF										
Overall (Group I, II, III)	MMP-9_serum		IL-6 in CF		IL-6_serum		TWEAK in CF		TWEAK in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.71	<0.001*	0.77	<0.001*	0.73	<0.001*	0.7	<0.001*	0.75	<0.001*
MMP-9 in serum										
Overall (Group I, II, III)	IL-6 CF		TWEAK in CF		TWEAK in serum		MMP-9 in CF		MMP-9 in serum	
	rho	P value	rho	P value	rho	P value	rho	P value	rho	P value
	0.71	<0.001*	0.8	<0.001*	0.79	<0.001*	0.57	<0.001*	0.74	<0.001*

*Significant at p value <0.001

activation, increased levels in target tissues and its function as a novel pathogenic mediator by amplifying inflammation, promoting tissue damage and impede endogenous repair mechanism in inflammatory disease which are associated with infiltration of inflammatory cells or activation of residents innate immune cells (Grossi SG 1998). Hence, the increased levels of TWEAK in subjects with stage III grade B Periodontitis and type 2 DM indicated its dual role in inflammatory conditions.

Although the study included equal number of males and females in each group of a specified age ranging from 25-60 years; the influence of age of the subjects on expression of the biomarkers cannot be nullified. The cross-sectional nature of the study also does not help to analyze the change in the levels of biomarkers over a period of time. The effects of periodontal therapy on the subjects and the concentration of these biomarkers following it were also not identified. The European Federation of Periodontology and International Diabetes Federation stated that Periodontitis has a significant impact on diabetes control, incidence and complications. Chair side kits can be refined for easier and faster diagnosis using these molecular markers. Therefore, Multicenter interventional studies with large sample size should be carried out to find the role of these markers of inflammation in Periodontitis and diabetes and also its relationship with poor glycemic control.

Conclusion

With the limitations of the present study, it can be postulated that there is an increased concentrations of TWEAK, IL-6 and MMP-9 in CF and serum from health to stage III Grade B Periodontitis to stage III Grade B Periodontitis with type 2 Diabetes. The markers have found to play a role locally at the site of tissue injury as in the case of Periodontitis and is also expressed in systemic conditions like Diabetes Mellitus. Hence the markers (TWEAK, IL-6 and MMP-9) can be considered as a potential inflammatory marker of Periodontitis and DM. These markers can also be valuable in detecting high risk individuals with Periodontitis and systemic diseases. Further multicenter, longitudinal, prospective studies must be carried out to confirm these findings and for better understanding of the possible roles of these molecular markers in the pathogenesis of periodontal diseases and diabetes.

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Latest Trend in Soft Tissue Evaluation Using Digital Technology

Yun-Jeong Kim DDS, MSD, PhD[1,2], Young Ku DDS, MSD, PhD[2]

[1] Department of Periodontology, Seoul National University Gwanak Dental Hospital, Seoul, Korea
[2] Department of Periodontology & Dental Research Institute, Seoul National University, Seoul, Korea

Introduction

The first step in dental digitalization is to reproduce the situation in the oral cavity with accurate data. Several clinical studies have attempted to create models and data or to use cone beam computed tomography (CBCT) images to measure the dimensions of the periodontal tissues, including soft and hard tissues. However, direct intraoral scanning can reduce the risk of errors associated with impression-model fabrication and obtain more precise contours of the tissues with higher resolutions.

Numerous studies have already shown that intraoral scanning can achieve comparable or better results in terms of accuracy, convenience, and efficiency compared to conventional impression taking. (Carbajal Mejia *et al* 2017 ; Guth *et al* 2017 ; Renne *et al* 2017 ; Park *et al* 2016) However, it is difficult to accurately represent the appearance of movable soft tissues, including the alveolar mucosa, even with a digital impression. Nevertheless, compared to the pressure associated with conventional methods, the shape of the gingival margin and interdental papilla can be reproduced without pressure; additionally, undercuts or structures under orthodontic brackets or under undercuts can be recorded, and the color of the gingiva can be identified through this intraoral scanning. (Park *et al* 2016 ; Ko *et al* 2020 ; Kim *et al* 2016 ; Kim *et al* 2021)

Therefore, in this study, the history of soft tissue data analysis obtained through digital impressions and the latest trends in analysis methods were reviewed, and the process of measuring the thickness of the gingiva and the three-dimensional (3D) volume of the alveolar ridge was discussed. Moreover, it was attempted to confirm that it can be applied to periodontal treatment by confirming and comparing the volume change of soft tissues before and after tooth extraction or surgery using digital scanning data.

Attempts to accurately measure the gingival thickness and correlation analysis of periodontal tissue dimensions

In a new classification, variation in the clinical appearance of mucogingival tissues has been called the periodontal phenotype, which is accepted as a concept encompassing the gingival biotype, bone morphology, and tooth dimensions(Jepsen *et al* 2018). It has long been known that gingival biotypes, including gingival thickness and keratinized tissue width, reflect underlying alveolar bone structures(Ochsenbein and Ross 1969). “Thin scalloped” gingiva is associated with a higher risk of gingival recession after immediate implant placement and poor soft tissue healing after crown lengthening surgery. (Evans and Chen 2008 ; Pontoriero and Carnevale 2001) These issues may be explained by several studies that reported a weak to moderate correlation between the thickness of the underlying bone and that of the overlying gingiva.(Fu *et al* 2010 ; Stein *et al* 2013) However, relatively fewer studies have investigated this issue due to the lack of standardized techniques for measuring hard and soft tissue thickness.

The simplest method for assessing soft tissue thickness in the clinic is to determine the degree of visibility of the periodontal probe contour through the soft tissue, while probing the buccal gingival sulcus.(De Rouck *et al* 2009) Measurement methods using a caliper on the model, invasive probing using endodontic needles, and non-invasive ultrasound devices have also been proposed. The method of measurement using CBCT with a marker attached to the gingival surface is also widely used. (Januario *et a*l 2011) However, the ability of these techniques to unambiguously determine the thickness of a specific site is limited; furthermore, the results were controversial, and the delineation between thick and thin biotypes was inaccurate.

To overcome the limitations of the above-mentioned methods such as invasiveness and inaccuracy, we devised a method to measure the thickness of the hard and soft tissues in a specific area through direct intraoral scanning and superimposition. (Figure 1,2) As a result of applying this to the maxillary anterior region, the gingival thickness at the alveolar crest level revealed a positive correlation with the

thickness of the underlying alveolar bone plate ($P<0.05$), although this correlation was not significant at identical depths. Gingival thickness at or under the alveolar crest level was not associated with the clinical parameters of the gingival features, such as the crown form, gingival scallop, or keratinized gingival width.(Kim *et al* 2021)

Dimensional ridge contour measurements change after tooth extraction

Following tooth extraction, the periodontal tissue surrounding the extraction socket undergoes structural changes including dimensional and radiographically visible changes. Alveolar bone resorption and remodeling occur within the alveolar process, and the buccal bone plate, with a high ratio of bundle bone, shows significant bone loss when compared to the lingual bone.(Araujo and Lindhe 2005 ; Schropp *et al* 2003) In most cases, the degree of horizontal reduction of the alveolar bone is greater than that of vertical reduction.(Tan *et al* 2012) Significant functional and esthetic limitations are associated with a prosthetic restoration, such as an implant, placed in soft tissue damaged due to tooth extraction. Various procedures have been attempted to minimize alveolar bone resorption and preserve the volume of periodontal tissue.

Alveolar ridge preservation (ARP) is a widely used procedure to reduce physiological resorption after tooth extraction and facilitate restorations for tooth replacing. (Avila-Ortiz *et al* 2019) Immediate grafting of extraction sockets serves as a scaffold for bone formation and reconstruction of ridge contours and minimizes dimensional loss, an inevitable consequence of soft and hard tissue remodeling.(Ben Amara *et al* 2021 ; Canullo *et al* 2022) Numerous studies support the superiority of ARP, compared to spontaneous healing, in attenuating the horizontal and vertical reduction of post-extraction ridges for periodontally damaged sockets as well as for intact sockets.(Cha *et al* 2019 ; Lee *et al* 2021) In particular, the reduction of bone atrophy by preserving the ridge is remarkable in premolars with low esthetic importance and anatomical advantages. (Schnutenhaus *et al* 2018 ; Hammerle *et al* 2012)

In the case of conventional model fabrication using an impression material, various errors inevitably occur when measuring the thickness or volume of soft tissue because its shape and volume can change even with slight pressure. Therefore, in a previous study, the 3D images obtained through intraoral scans were superimposed to examine the contour changes according to the extraction healing process and were compared with the results of previous similar studies in which plaster models were scanned and overlapped. (Thoma *et al* 2020) Although only soft tissue contours can be scanned, it has been found through several studies that the thickness of soft tissue can be accurately measured by matching the results of intraoral digital impressions with the CBCT images of the alveolar bone.(Kim *et al* 2016)

In our recent clinical study, from among 16 patients with a mean age of 32.19 (range: 19–61) years, 32 bilateral premolars were extracted, and ridge preservation was performed on one side; a horizontal change in width was observed. (Figure 3)

To measure the change in gingival width over time, all

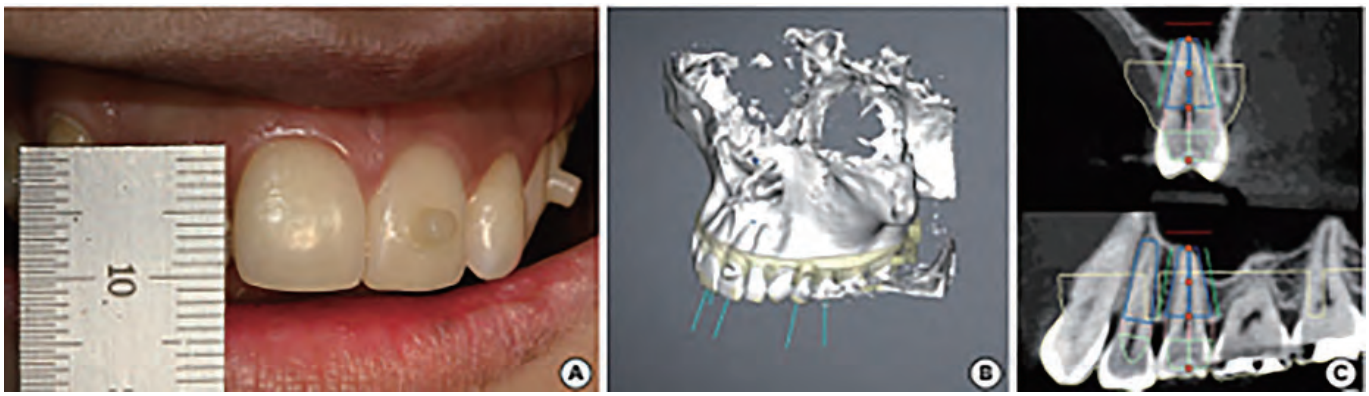

Figure 1. Three radiopaque, cylindrical fiducial markers, measuring 2 mm in diameter by 2 mm in height, were attached to both maxillary premolars and 1 incisor (A). Image reconstruction for visual analysis was performed using Platon software (Ezplant) to automatically superimpose the images (B, C).

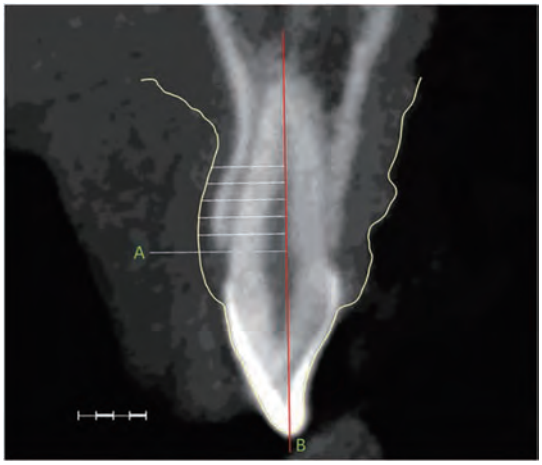

Figure 2. Para-axial slice at the mid-buccal aspect of the lateral incisor. The gingival outline obtained from a scanned file is marked as a yellow line. Thickness measurements at 1–5 mm from the alveolar crest (A), and perpendicular to the root axis (B).

3D-scanned images were superimposed with an automatic best-fit alignment option, using neighboring teeth as reference structures (Geomagic Verify 16; 3D Systems, Rock Hill, USA). Using another software (Rapidform 2006; INUS Technology, Seoul, Korea), a coronal section was selected to divide the extraction socket area into two equal parts in a previously known manner.(Ben Amara *et al* 2021 ; Aimetti *et al* 2018) At the baseline, a reference line was drawn through the buccal and lingual gingival margins. The horizontal ridge width (HW) was measured 2 and 4 mm (H2 and H4) below the reference line in all three STLs, similar to previous studies(Thoma *et al* 2020), and the change in HW over time was also measured. Vertical changes were measured at the center of the ridge, perpendicular to the reference line. After superimposing the three scan results, horizontal change on the buccal and lingual sides was measured by height, and the vertical change at the center of the extraction site was also measured. (Figure 4)

The volumetric change in the alveolar ridge is measured by superimposing 3D models of the extraction socket over time. Regions of interest (ROIs) were established on the buccal and lingual sides of each socket. According to a number of studies, the ROI is created by drawing straight lines perpendicular to the occlusal surface at the mesial and distal interdental papilla of the adjacent tooth to be extracted and setting the horizontal line to 1 and 3 mm below the gingival margin. Thus, the distance between the ROIs was measured, surfaces of the buccal and lingual ROIs were extracted and connected to create a 3D polygon, and then the volume was measured.

According to a pilot study(Kim *et al* 2021) published by analyzing the data of 9 subjects, the horizontal ridge contour during the healing process of healthy mandibular premolar tooth extraction showed a tendency to decrease over time, and a greater change was observed as the height of the measurement point was closer to the reference gingival margin.

Repeated measures analysis of variance (rm-ANOVA) was applied to evaluate the effects of ridge preservation and time on each outcome. In addition to the comparison of the linear measurements, the average distance between gingival surfaces and the change in the volume of the 3D polygon connecting the ROIs were examined. The effects of ridge preservation were not evident. When measuring linear cross-section or quantifying three-dimensional distance and volume change, there was no significant difference in the change over time between the two groups.

Latest digital technology based on scanning to measure the volume change of soft tissue

In a recent 2021 European Association for Osseointegration (EAO) consensus report (Thoma *et al* 2021), it was recommended that linear soft tissue changes be measured preferably on superimposed digital surface scans to assess profilometric soft tissue changes. Despite this trend of digitalization in dentistry, workflows for generating and analyzing STL files and accurate metrology methods for observing volumetric changes have not yet been systematically evaluated. Moreover, no standard recommendations or guidelines exist on defining an ROI to evaluate volume changes using a 3D image analysis software, which inevitably causes errors in the work process and evaluation of results.

A 3D digital model can be created by scanning directly within the oral cavity or by scanning a study model fabricated using conventional methods. Various types of software are used to superimpose and analyze the obtained STL files. Key outcome measures for reporting volume change can be determined by measuring the volume change in mm^3 (Figure 5), the average distance between surfaces (Figure 6), or the linear dimensional change of the apico-coronal cross-section.

(Figure 4)

Most recent studies have concluded that the direct digital impression method has an accuracy similar to that of indirect model scanning methods. The intraoral scanner not only allows the operator to perform the impression process quickly and comfortably, but also reduces patient discomfort, pre-visualizes the ROI in 3D and provides opportunity to quickly re-scan the missing areas. This is in contrast to the conventional impression method, which requires repeating the entire procedure in case of a mistake.(Di Fiore *et al* 2018)

On the other hand, it should be noted that the accuracy of intraoral scanners greatly depends on the skill of the operator, type of intraoral scanning system, and range of the scanning area. Therefore, operator calibration is necessary, and it is desirable for the examiner for 3D measurements to be calibrated or be blind to the intervention. (Tavelli *et al* 2021)

It is known that the accuracy of impressions decreases with increasing scan area. Therefore, many studies tend to target only small areas of interest rather than attempting full-arch scanning. (Schmidt *et al* 2020) Different software are available for the superimposition of digital files and 3D analysis results, with most studies using an automated or semi-automated method of superimposition. Depending on the type of software with different programs, the quality of the resultant STL file can affect the results. The prevailing opinion is that it is important to trim the digital cast to achieve a reliable and accurate superimposition. It is also important to recognize that mastering software functions for skilled digital measurements entails a learning curve.

Attempts have been made to use 3D analyses rather than

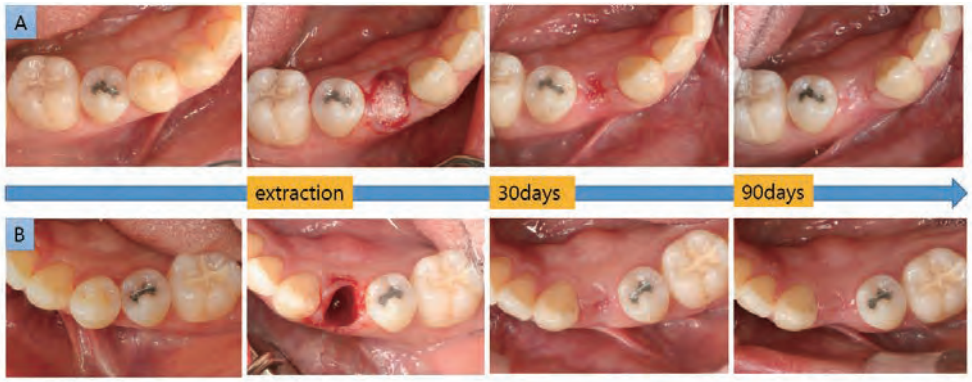

Figure 3. Sequence of clinical photographs of each clinical visit obtained before extraction, 30days and 90days after extraction of alveolar ridge preservation treatment group (A) and control (B).

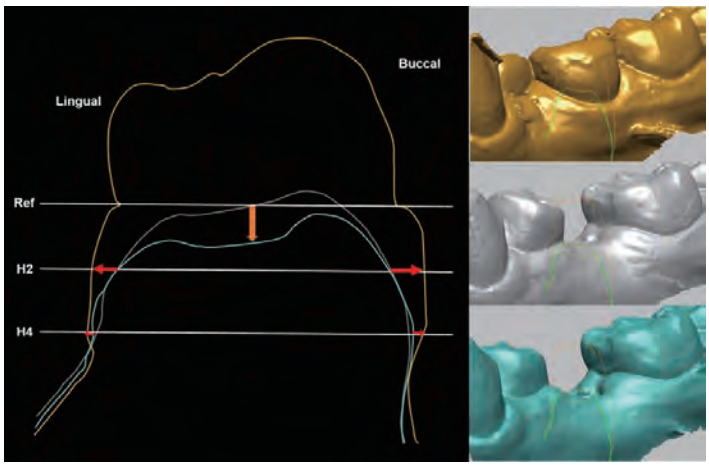

Figure 4. Cross-sectional view of the region of interest.
The contour of the yellow model (before tooth extraction), white model (30 days after tooth extraction), and blue model (90 days after tooth extraction) were superimposed according to the adjacent teeth. The reference line was drawn through the buccal and lingual gingival margins. Horizontal ridge width (HW) was measured (red) in all three STLs at 2- and 4 mm (H2 and H4) below the reference line. Vertical change in the ridge was measured perpendicular to the reference line (orange) at the center of the ridge.

Figure 5. Volumetric measurements in mm3. The three-dimensional (3D) volume change can be compared by extracting regions of interest (ROIs) defined by the same border line from the buccal and lingual surfaces and calculating the volume of a 3D structure connecting them. (Geomagic Verify 16)

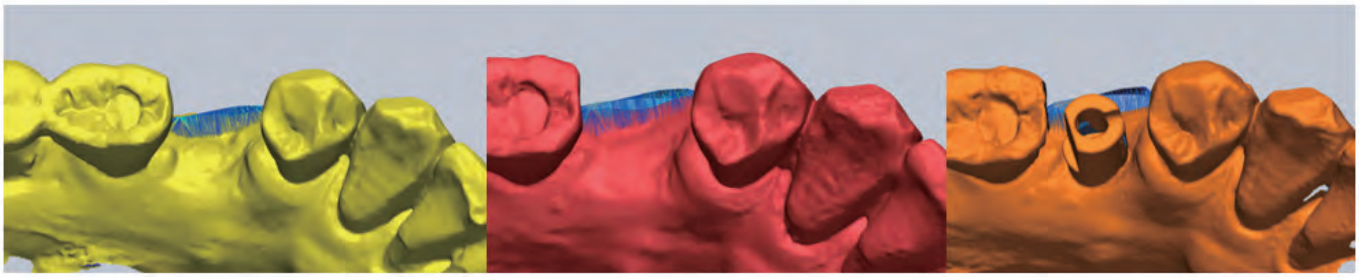

Figure 6. Mean distance between the surfaces. The method of setting the regions of interest (ROIs) on the buccal gingiva and measuring the average of the distance between the surfaces over time is widely used to observe volumetric changes. (Geomagic Verify 16)

cross-sectional linear measurements to determine the total volume change before and after treatment. Any change in the buccolingual direction perpendicular to the labial plane at the selected reference point can be expressed as the average distance between the two analysis planes. (Tian *et al* 2019) By setting the volumetric ROI to be cut and measured in the same way in all digital models, the total volume change can also be directly derived in mm^3.(Borges *et al* 2020)

Conclusion

Various attempts have been made to three-dimensionally analyze the change in the soft tissue thickness and alveolar ridge volume using digital equipment such as an oral scanner and various software packages.

However, interventional studies comparing this method with other approaches to assess volume change are lacking, and the digital impression technique or software type, superimposition method, and ROI setting criteria vary; therefore, it is necessary to establish standardized analysis methods and guidelines.

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Efficacy of Crown Lengthening Surgery and Botulinum Toxin A in Treatment of Gummy Smile

Thuy Thu Nguyen[1], Viet Tran Ho[1], Hao Trong Nguyen[2], Vu Hoa Anh Dien[1], Anh Quynh Nguyen-Ho[1], Hien Thi Minh Nguyen[1], Nam Cong-Nhat Huynh[1]

[1] Faculty of Odonto-Stomatology, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam

[2] Ho Chi Minh City Hospital of Dermato-venereology, Ho Chi Minh City, Vietnam

Introduction

Dentistry is a medical specialty that focuses on improving one's smile, and the establishment of clinical treatment plan for a beautiful smile remains difficult. The degree of gingival display, the proportion of anterior teeth, and the gum line are the factors that assist the dentist plan practical and effective treatment. On the other hand, the society's demand for beauty is rising, and more people seek cosmetic enhancements, such as gummy smile (GS) repair. As a result, the goal of this new study was to analyze the efficacy of a combination of clinical dental crown lengthening surgery and Botulinum toxin A (BTX-A) injection in GS treatment using clinical and photographic evaluation.

GUMMY SMILE

According to a recent research, when smiling, the greatest amount of gingival display allowance is 3 mm(Oliveira *et al* 2013). Otherwise, it can be classified as a GS, which is less appealing(Dym and Pierre 2020). According to a study conducted by Horn *et al* in 2021, the incidence of GS was about 31%.

ETIOLOGY

A gummy smile is often caused by a combination of several variables working at the same time (Tjan and Miller 1984). Gummy smile biomechanics also appears to involve the combined impacts of teeth, gums, skeletal structure, and muscles. No single procedure can guarantee that the patient's aesthetic needs are met.

TREATMENT

Gummy smiles can be treated by using a variety of interventions, including orthodontics, orthognathic surgery, osteotomy/osteoplasty, and gingivectomy. While orthodontic treatment is costly and time-consuming, it is also difficult and invasive. When paired with altered passive eruption and the correction of hypermobile lip muscles, gingivectomy and smile line relocation address gummy smile cases(Kokich 2006 ; Seixas *et al* 2011). Both treatments, however, are not only intrusive, but they also create worry and agony after surgery.

CROWN LENTHENING SURGERY

Lidocaine 2% and 1:200,000 epinephrine were used to anesthetize the patients locally. Bone sounding was used to determine the distance between the gingival margin and the bone crest. It's usually 3 mm on the mid-buccal and mid-lingual sites, and 4.5-5.0 mm on the proximal sites. Before marking the bleeding spots using a periodontal probe and a surgical template, the height of keratinized gingiva was re-evaluated (must be 3 mm) (Garber and Salama 1996 ; Fradeani 2006).

The first incision was made 45^0 apically to the tooth axis as the internal bevel gingivectomy. Following that, a second incision was made along the gingival sulcus. The gingiva was removed from the root surface, and the periodontal curettes were used to clean the residual tissue attached to the root. A minimum of 4-5 mm alveolar ridge height was exposed, and a full-thickness flap was performed from the gingivectomy border to the mucogingival junction. A 2 mm diameter carbide bur was used to trim the alveolar bone in order to establish a proper supra-crestal attachment (biological width) , which was 2.5 mm at least from the anterior teeth in all aspects (from cemento-enamel junction to the crest of bone). Finally, non-resorbable 5-0 polyamide sutures were employed.

BOTULINUM TOXIN A

BTX-A has been developed for use in other therapies and has been proven to be a safe substance in the treatment of

gummy smiles. Excessive gum display can be reduced by injecting BTX-A into the upper lip levator muscles, such as the levator labii superioris, levator labii superioris alaeque nasi, and the zygomaticus major and minor, in appropriate instances(Sundaram *et al* 2016 ; Hwang *et al* 2009).

EFFICACY of Crown Lengthening Surgery and Botulinum Toxin A COMBINATION in Gummy Smile TREATMENT

For patients with altered passive eruption, surgical treatment of gummy grins is beneficial. Furthermore, this surgical approach assisted in the restoration of the correct clinical crown height as well as the adjustment of gingival asymmetry (Maynard Jr and Wilson 1979). However, treating gummy smiles remains a difficulty for clinicians. Gingival recession can result from a considerable amount of bone reduction, while insufficient bone grinding can lead to recurrence.

To prevent recurrence, in the current study, the flap was moved at least 2 mm away from the bone crest, which is the minimal space for biologic width. Clinical crown length, on the other hand, was dramatically improved after two months as compared to the initial status. Furthermore, gingival display decreased considerably with $p \leq 0.05$ from T1 (2 months after surgery) to T2 (2 weeks after BTX-A injection) (1.9 mm and 4.2 mm, respectively). As a result, 3 days after injection, BTX-A was efficient in decreasing gingival display. The outcome agrees with the findings of Ahmet's study in 2020.

BTX-A reached its maximal effect two weeks after injection (T2) (Suber *et al* 2014). The average gingival display at T2 was 1.5 mm. Before the injection of BTX-A, it was 3.5 mm (T1). There were no side effects from BTX-A. Polo *et al* (2008) had comparable results. The extent of gingival display decrease varies among researches and could be attributed to sample size, inter-surgeon variation, and

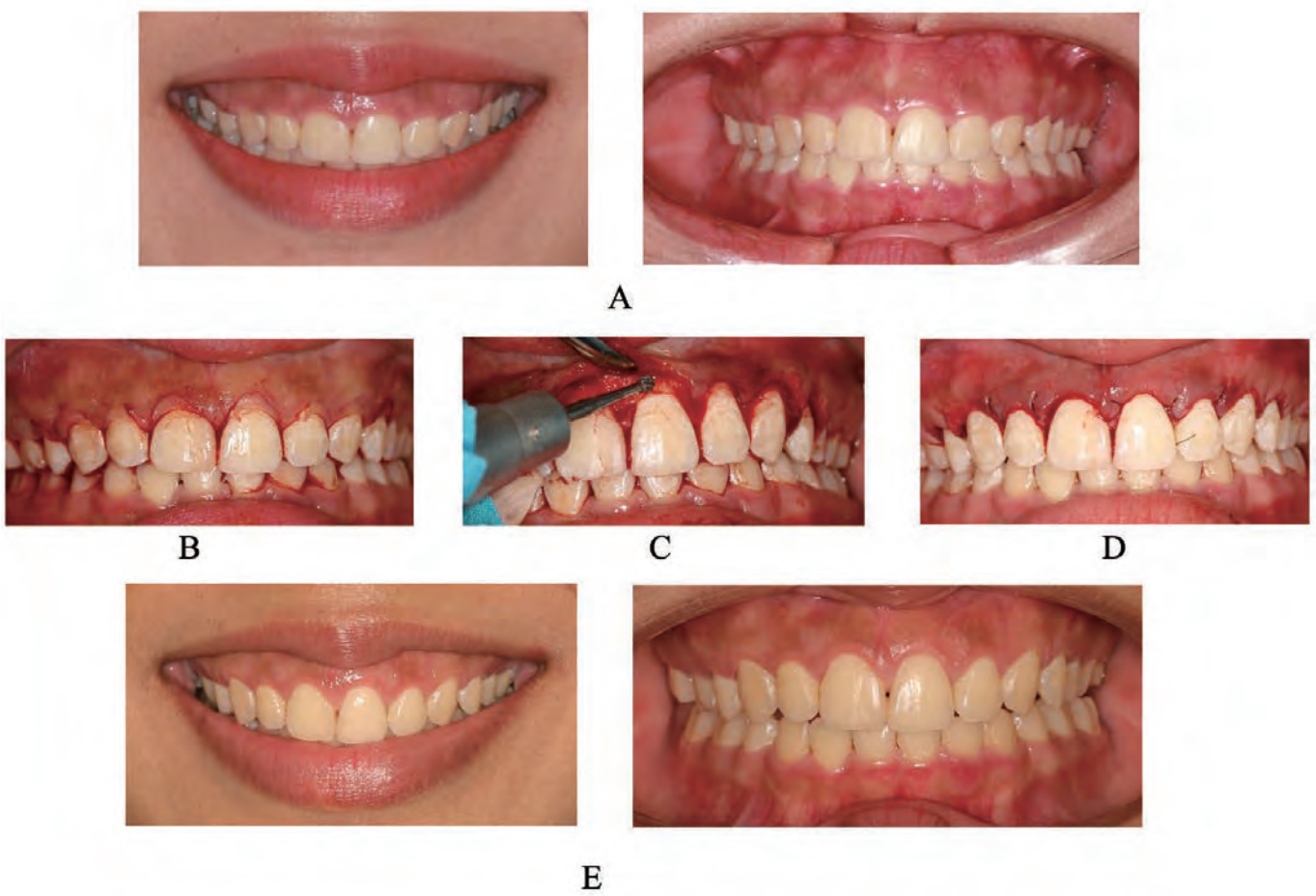

Figure 1. A. Before crown lengthening surgery. B. Gingivectomy on the gingiva of teeth 15 – 25. C. Osteoplasty on the alveolar ridge of teeth 15 – 25. D. Sutures. E. Two weeks after suture removal.

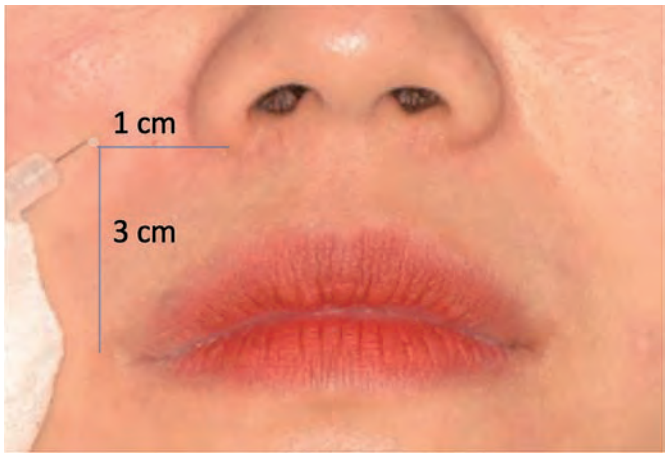

Figure 2. BTX-A injection

difference in metabolism among races living in different

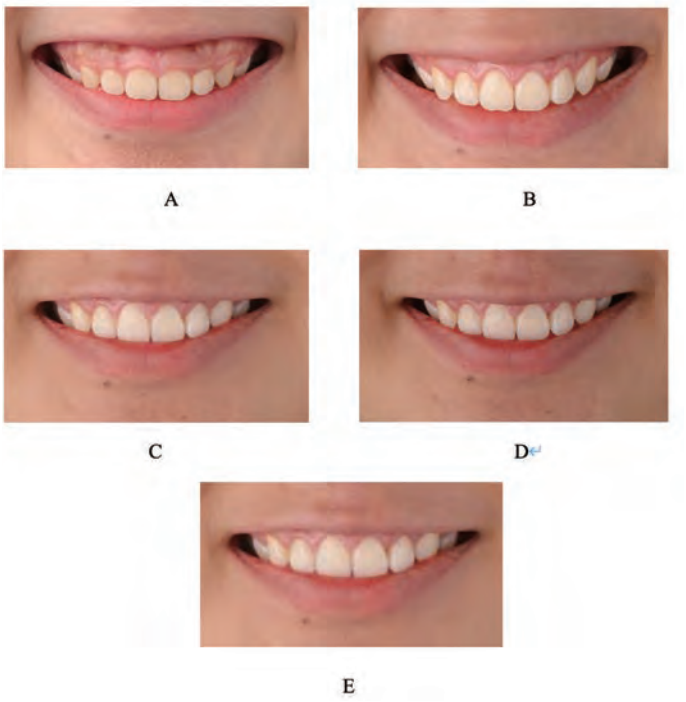

Figure 3. A. Before intervention B. After clinical crown lengthening surgery. C. 3 days after BTX-A injection. D. 2 weeks after BTX-A injection. E. 2 months after BTX-A injection.

geographical places. In patients with mild gummy smile, we could fairly infer that BTX-A successfully reduced gingival exposure.

BTX-A injections are minimally invasive treatments. However, the results are only transient. After 3-6 months, the efficacy of BTX-A gradually diminishes. If the patient wants to maintain that result, he or she must inject BTX-A again.

CONCLUSION

By extending the crown height and modifying the width and length ratio of the clinical crown to a good aesthetical standard, clinical crown lengthening surgery dramatically reduced gingival display when smiling. BTX-A injection improved the outcome, as evidenced by observations made three days, two weeks, and two months after injection. The levator labii superioris, levator labii superioris alaque nasi, zygomaticus minor, and zygomaticus major muscles were all considered safe injection sites. As a result, we believe that BTX-A is an effective supplementary treatment for individuals who have had anexcessive gingival display.

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Healing of Peri-implant Bone Defects in Sheep Femur Using Nanosilver-Modified Bone Xenograft – A Pilot Study

WJ Duncan, D Abdelmoneim, E Elahi, GC Porter, KC Li, DE Coates

Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand.

Keywords: Dental implant, Guided bone regeneration, xenograft, nanosilver, animal model

Abstract: Guided bone augmentation to fill peri-implant defects may be required during immediate implant placement if there is a mismatch between tooth socket size and implant diameter. However, infection of the bone-grafted site may be a complication, and there are increasing problems of bacterial resistance to the antibiotics used to treat these infections. MoaBone® (MB) is a bovine bone xenograft (BBX) derived from New Zealand disease-free cattle, widely used for ophthalmic grafting, whose properties we modified (optimised MoaBone, OMB). We then doped the OMB using antibacterial lipoic-capped silver nanoparticles (AgNP) with encouraging results in a rabbit cranial critical-size defect model. The objective of the current pilot study was to examine peri-implant bone healing in a sheep femur model. Methodology: In 4 sheep, two x 8.5mm diameter, 4mm deep circular defects were trephined in the femoral epicondyle of both posterior legs. Narrow titanium implants (Straumann®), 3.3mm diameter x 8mm to 12mm length, were installed into the middle of each defect. The 2.6mm gap around the coronal portion of each implant was filled with either OMB, OMB with AgNP, commercially-available BBX (BioOss®; Geistlich Pharma Ltd) or left unfilled. All defects were covered with a resorbable collagen membrane (Osseoguard Flex®, Zimmer). Two sheep were euthanised at each timepoint after 8 and 12 weeks. Samples were analysed using non-demineralised resin-embedded histology. Results: OMB with and without AgNP demonstrated equivalent bone fill to each other and to BioOss®; all grafted sites showed more bone fill than un-grafted controls. Results were not statistically compared in this small pilot study. Conclusions. Both modified OMB BBXs showed equivalent results to commercially available BBX (BioOss®). The addition of AgNP did not impair bone healing. This pilot study warrants progressing to larger-scale preclinical trials including intra-oral models with infected sites.

Introduction

The techniques first described as "guided tissue regeneration" (GTR)(Pontoriero *et al* 1988) and subsequently as guided bone regeneration (GBR)(Dahlin *et al* 1988), have evolved over forty years from non-resorbable Teflon to resorbable synthetic or collagen membranes(Hürzeler *et al* 1997) accompanied by bone replacement grafting materials(Zitzmann *et al* 1997). The history of this development has recently been comprehensively reviewed(Scantlebury and Ambruster 2012). Different grafting technologies and materials may be used contemporaneous with installation of a dental implant into a fresh extraction socket when there is a miss-match between implant and tooth socket diameters (Botticelli *et al* 2003 ; Araujo *et al* 2011 ; Ghanem 2015), or to cover exposed implant surfaces when there is a bone deficiency(Hämmerle *et al* 2012) or for treatment of peri-implantitis if circum-implant bone has been lost due to the infective-inflammatory disease peri-implantitis (Schwarz *et al* 2006). However, the risk of graft or implant failure due to infection after immediate implant placement remains unclear, with two recent systematic reviews reaching conflicting conclusions (Chrcanovic *et al* 2015 ; de Oliveira-Neto *et al* 2019). In addition, recent systematic reviews suggest a lower failure rate when antibiotics are used concomitant with implant placement (Kim *et al* 2020 ; Salgado-Peralvo *et al* 2021). However, world-wide there is recognition that antibiotic resistance is an increasingly significant problem (Ventola 2015) and practitioners are now advised to follow the principle of antimicrobial stewardship and to avoid the routine use antibiotics following implant treatment in healthy patients (Park *et al* 2018).

For these reasons, there is increasing interest in the use of non-traditional antibacterial agents, for example colloidal silver and more recently, silver nanoparticles (AgNPs)(Rai *et al* 2012 ; Dos Santos *et al* 2014). AgNPs are increasingly being used to prevent microbial colonization and infection around clinically-applied titanium biomaterials (Croes *et al* 2018 ; Gunputh *et al* 2018) and have shown potential for treatment of oral biofilms in many aspects of dentistry (Fernandez *et al* 2021 ; Skóra *et al* 2021).

The development of grafting materials and techniques follows a predictable pathway, from benchtop and *in vitro* testing, to *in vivo* analysis in different preclinical animal models, before finally proceeding to human clinical trials (Duncan and Coates 2022); understanding the relationship

between these steps is often critical to interpreting the success of a novel intervention (Duncan *et al* 2022). Our research group has developed a modified form of deproteinized bovine bone xenograft spheres that are currently used for ophthalmological grafting (MoaBone® M-Sphere®, Molteno® Ophthalmic Ltd, Dunedin, New Zealand)(Duncan *et al* 2020). Our novel bovine xenograft was developed to reduce the rapid remodeling of MoaBone® that we found during maxillary sinus grafting in an animal model(Smith *et al* 2018) and has now undergone optimization using a combination of 160°C heat and pressure followed by gamma irradiation steps. This optimized MoaBone (OMB) has undergone rigorous testing, both *in vitro* and *in vivo* in a small animal trial, with encouraging results (Abdelmoneim *et al* 2022 ; Porter *et al* 2022). Simultaneously, we have also developed technology using lipoic-capped AgNPs as alternative antimicrobial agents (Cotton *et al* 2019 ; Porter *et al* 2021) with which we have doped our OMB xenograft.

The aim of the current pilot study was to compared two novel graft materials, OMB and OMB with added silver nanoparticles (OMBAgNP), against the commercially-available bovine bone xenograft BioOss®, for regeneration of bone within a peri-implant critical size defect in the femurs of sheep. Our hypothesis was that (1) the test grafts OMB and OMBAgNP would demonstrate equivalent osseous healing to BioOss® and (2) the test grafts would not impede osseointegration of dental implants in this unloaded, non-oral animal model. To the best of our knowledge, we are the first to report the *in vivo* osseous healing response and osseointegration of titanium implants adjacent to AgNP-doped bone xenografts.

Methods

This study was approved by the Otago University Animal Ethics Committee (Animal Use Protocol AUP 21-115) in accordance with New Zealand Regulations (New Zealand Animal Welfare Act 1999). The ARRIVE guidelines for reporting animal research were followed. Four Romney-cross ewes over 3.5 years of age were used in this study. Prior to the beginning of the study, all animals underwent thorough health screening to exclude footrot and pregnancy and received appropriate immunisations and treatment to control parasites. All sheep were required to be in good health and weigh greater than 60 kg.

This was a pilot study with two healing periods and four treatment arms including a control (non-grafted) group. The animals were divided into 2 groups of 2 sheep each, with one group allocated to each of the healing periods (8 weeks and 12 weeks).

Grafting materials

The two test materials used were optimised MoaBone® (OMB) and OMB with silver nanoparticles (OMBAgNP). The production of OMB has been fully described and produces a grafting material with acceptable mechanical properties, resorption rates and surface characteristics (Cotton *et al* 2019 ; Porter *et al* 2021). Production of alpha-lipoic acid capped-AgNPs have also been previously described results (Abdelmoneim *et al* 2022 ; Porter *et al* 2022).

Briefly, prion-free cancellous bovine femoral bone blocks measuring 25mm x 25mm x 25 mm were degreased and cleaned by boiling, rinsed in water at 80°C and centrifuged by the manufacturer (Molteno® Ophthalmic Ltd, Dunedin, New Zealand). The blocks were then reduced to 8mm x 8mm x 8mm cubes using a Struers Accutom-50 cutting machine (Struers, Denmark). Thermal processing was conducted in a custom-made cylindrical stainless-steel vessel containing distilled water, heated at a rate of 4-6 °C/min and held for 2 h at 160 °C, followed by a 5 minute cooling period. Blocks were rinsed with distilled water, air dried for 24 h in a sterile environment at room temperature (RT), and immersed in 1% sodium hypochlorite solution under vacuum at RT for 24 h. This was repeated four times with centrifugation between bleaching cycles. The bone graft material was then rinsed with reverse osmosis (RO) water and air-dried. Bone blocks were particulated and sieved to obtain particles between 0.25 mm – 2 mm, which were gamma irradiated at 25-32 kGy (MSD Animal Health, Gamma Department, New Zealand).

Alpha lipoic acid capped-AgNPs were prepared by combining 40 ml docusate sodium salt solution (≥ 96%, Cat. No. 86140, Sigma Aldrich, Missouri, USA) with 0.33 M heptane (Cat. No. H350-1, Fischer Scientific, New Hampshire, USA), stored in two separate flasks. Two microemulsions (μEm) were formed by adding (1) an aqueous solution of silver nitrate (AgNO3; Cat. No. 10224350; Fisher Scientific, New Hampshire, USA) (1.6 ml, 0.13 M) dropwise with stirring to one flask, and (2) an aqueous solution of sodium borohydride (NaBH4 crystalline

98– 99%, Cat. No. ICN10289425, Fischer Scientific, New Hampshire, USA) (1.6 ml, 1.84 M) dropwise with stirring to the second flask. The flasks were placed in separate ice baths, and the second μEm added dropwise with continuous stirring to the first, in darkness, for up to 24 h. Subsequently, alpha lipoic acid (0.08 mM, dissolved in 0.25 ml ethanol) was introduced and the μEm stirred for an additional 2 min. A 1:1 methanol/acetone mixture was added to 40 ml of the combined μEm until phase separation was observed and a dark-coloured interface containing the AgNPs formed between the two phases. The particles were extracted and washed 3 times with ethanol with centrifugation at 6000 g for 5 min, and resuspended in 1–6 ml of deionised (DI) H2O adjusted to pH 10 with anhydrous ammonia. The resulting yellow-brown colloidal suspension was centrifuged twice at 16000g for 45 min and the final supernatant collected. AgNP size was typically <10 nm in size, with a narrow PDI <0.3 and a high concentration of approximately 3000 μg/mL[29]. AgNP stock (concentration 1110 μg /ml) was prepared and added within 4 weeks of production to the gamma irradiated OMB bone particles. The OMBAgNP particles were then air-dried at RT. The final concentration of AgNP per construct was 100 μg per gram graft material (Moussa *et al* 2021).

The control BBX consisted of BioOss® spongious bovine bone xenograft, of particles size 1-2mm (Geistlich Pharma New Zealand Ltd.)[31]. The membrane we used was Osseoguard Flex 30 x 40mm, a cross-linked resorbable bovine type I and III collagen membrane (Zimmer Biomet Dental, New Zealand).

Groups

Each animal had two defects created in each femur, giving a total of four defects per animal. All sites were consistently grafted with the same treatment: MoaBone® (MB), Moabone® with silver nanoparticles (OMBAgNP), BioOss® or None (un-grafted control). All implants were 3.3mm diameter and ranged from 8 to 12mm in length (Straumann New Zealand Ltd). The distribution of treatments and implants is shown in Table 1.

Surgical protocol

Surgery took place in the main animal operating theatre at Invermay Agricultural Research Centre in Mosgiel New Zealand. Full-scrub aseptic theatre techniques and sterile instruments were used at all times. All dental implants, membranes and bone grafts used in the study were provided pre-sterilised and sealed.

The animals received only water for 24 hours preceding general anaesthesia, along with antibiotics (Amphoprim®; Trimethoprim sulpha, subcutaneous injection 1 ml/15 kg, Virbac New Zealand Ltd., East Tamaki, Auckland). General anaesthesia was induced with intravenous Diazepam (Pfizer, New Zealand Ltd) 0.2 mg/kg and Ketamine (Baxter Healthcare Ltd) 2 mg/kg. The animals were restrained and placed in dorsal recumbency. After oral intubation and placement of a stomach tube, general anaesthesia was maintained using Isoflurane gas 2.5-3.5% to effect. For haemostasis, operative sites were infiltrated with local anaesthetic (Septodont Lignospan®, Lignocaine Hydrochloride 2% with Adrenalin 1:80000; Ivoclar Vivadent Ltd, New Zealand). The surgical approach used the femoral dental implant model discussed by Chappard et al. (1999) and subsequently modified by our team (see Figure 1)(Duncan *et al* 2018 ; Sharma *et al* 2016 ; Siddiqi *et al* 2016 ; Duncan *et al* 2018). The hind legs were shaved, the skin disinfected with povidone iodine and alcohol and then chlorhexidine 2 % in alcohol, and draped. Each femur was exposed by a medial approach from great trochanter to distal epiphysis via a skin incision 8-10 cm in length. The periosteum was incised and raised as a two-sided flap. Sequentially on the left and right femurs, two 8.5 mm diameter x 3 mm deep osteotomies were created using endcutting trephine burs (Meisinger, Neuss, Germany). In the middle of each defect, 3.5 mm implant osteotomies of 8 to 12 mm depth were created using the manufacturer's drills. Straumann implants were installed at low speed with copious chilled saline irrigation and coverscrews placed (Figure 2). The circum-implant "moat" defects were then grafted using MB, OMBAgNP, BioOss® or un-grafted ("None"). All defects were then covered with an Osseoguard Flex collagen membrane. The incisions were closed by layers with horizontal mattress and continuous suturing, first periosteum followed by fascia lata and muscle, using 3-0 Vicryl (Ethicon, Edinburgh, UK, Cat# J497H), followed by skin using 2-0 Maxon (Covidien, Dublin, Republic of Ireland, Cat# 88866267-51) as a continuous suture. Animals were extubated and recovered to a housing pen. For the three days following surgery, daily postoperative antibiotics and anti-inflammatory

medications were administered as follows: Caprofen 4 mg/kg (Rimadyl® injection 50 mg/ml, Zoetis, Mt Eden, New Zealand) and Amphoprim (Trimethoprim sulpha, Virbac New Zealand Ltd., East Tamaki, Auckland) 1 ml per 15 kg via subcutaneous injections. Food and water intake was closely monitored daily, as well as animal health and behaviour; any complications at surgical sites were examined and recorded. Subsequently the animals were transported to the research farm and allowed to graze freely with daily monitoring until the designated euthanasia date.

After 8 weeks and 12 weeks, N=2 animals per time point were returned to Invermay Agricultural Research Facility and anaesthetised following the same protocol as described. The carotid arteries were then exposed bilaterally by blunt dissection, ligated and cannulated, and the animals euthanised by anaesthetic Isoflurane overdose. Immediately after euthanasia, the jugular veins in the neck were severed and blood drained into a biohazard container; then 1 L per side of sterile saline with heparin (0.9% Sodium chloride, Baxter Healthcare Ply Ltd., NSW, Australia + 5000 IU heparin) was used to flush the vasculature. This was followed by 1 L per side 4% formaldehyde (BioLab Ltd., New Zealand) to perfuse and fix the tissues. The femurs were dissected and experimental sites separated.

Histology

Two samples each from the left and right sides were stored separately in 10% neutral buffered formalin for 1 week. Samples were transferred to 20% ethanol solution (Emsure®, LabSupply New Zealand) prepared fresh by combining RO water and 100% ethanol. Each sample was radiographed using a Nomad Pro 2 portable x-ray unit (Icona Ltd, Palmerston North, New Zealand) with Xios XG digital CMOS sensor connected to a laptop running Sidexis software (Sirona NZ Ltd). Samples were then assessed using μCT, the results of which are not reported here.

All specimens were then embedded without demineralisation. The protocol initially described by Donath and Breuner (1982) and later modified by Duncan (2005) was used for resin embedding. Briefly, specimens were dehydrated in ascending concentrations of ethanol (20, 40, 70, 90 and 100%) for two days per concentration, then transferred to a fume hood and placed into xylene solution (Ajax Finechem Pty Ltd, New Zealand) on a rotating platform. The samples remained in xylene for 4 days with two changes of solution. Specimens were rinsed with methyl methacrylate (MMA) (Methyl Methacrylate 99% stabilised, Acras Organics) and placed in MMA I solution and then MMA II solution for two days each on a rotating platform. MMA I is pure methacrylate; MMA II and III contained increasing concentrations of polymerisation initiator (Luperox® A75, 75% Benzoyl peroxide, Aldrich Sigma New

Table 1. Distribution of grafting materials and implant types

		Left femur		Right femur	
	Healing period	**Superior site**	**Inferior site**	**Superior site**	**Inferior site**
Sheep 1	8 weeks	BioOss®, SP SLActive 3.3x 12 mm,	OMBAgNP, SP SLActive 3.3x 12 mm,	None, SP SLActive 3.3x 12 mm	MB, SP SLActive 3.3x 12 mm
Sheep 2	8 weeks	BioOss®, TE SLA 3.3 x 10mm	OMBAgNP, TE SLA 3.3 x 10mm	None, TE SLActive 3.3x12mm	MB,TE SLActive 3.3x10mm
Sheep 3	12 weeks	BioOss®, SP SLActive 3.3x 10 mm	OMBAgNP, BL SLA 3.3 x 12mm	None, SP SLActive 3.3x 10 mm	MB, BL SLA 3.3 x 10mm
Sheep 4	12 weeks	BioOss®, BL SLActive 3.3 x 8mm	OMBAgNP, BL SLActive 3.3 x 12mm	None, BL SLA Roxolid® 3.3 x 12mm	MB, BL SLActive 3.3 x 12mm

Key:

SP = Standard Plus	SLA = large grit-blasted acid-etched
BL = Bone level	SLActive = large grit-blasted acid-etched, hydrophilic surface
TE = Tapered Effect	Roxolid® - 15 % zirconium 85 % titanium alloy
OMB = Optimised MoaBone	OMBAgNP = optimised MoaBone with silver nanoparticles
None = un-grafted control	

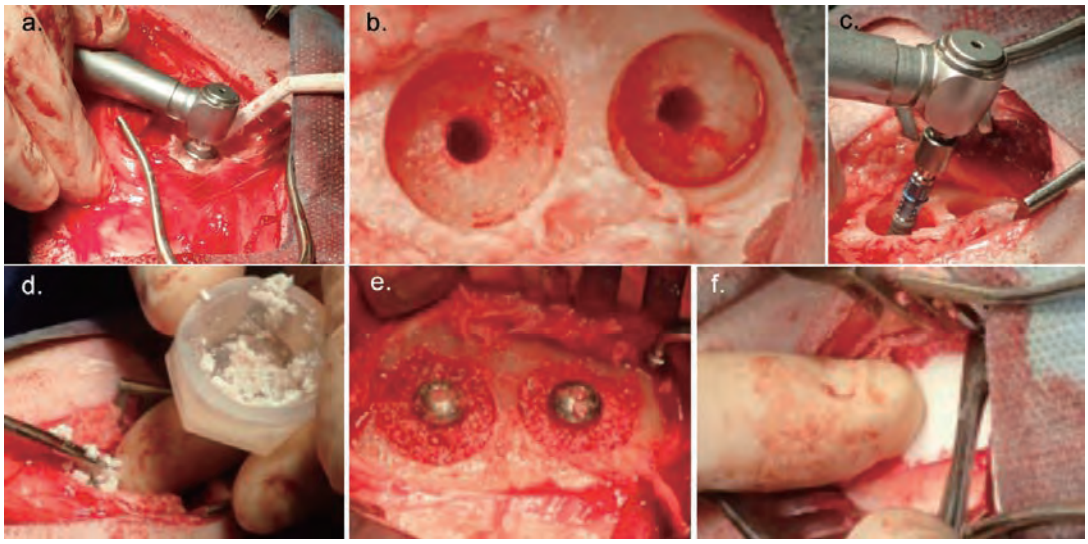

Figure 1. Surgical protocol for sheep femur. (a). Preparing bone defects in sheep femur. Using trephine and end-cutting ablative burs; defect measure Ø8.5mm x 3mm. (b). Two defects with central osteotomy drilled for dental implants. (c). Placing implant. (d). Placing bone graft. (e). Bone graft and implants: (Left) OMB optimized MoaBone bovine bone xenograft; (Right) Geistlich Bio-Oss® bovine bone xenograft. (f). Applying membrane Zimmer Biomet Osseoguard Flex resorbable collagen membrane.

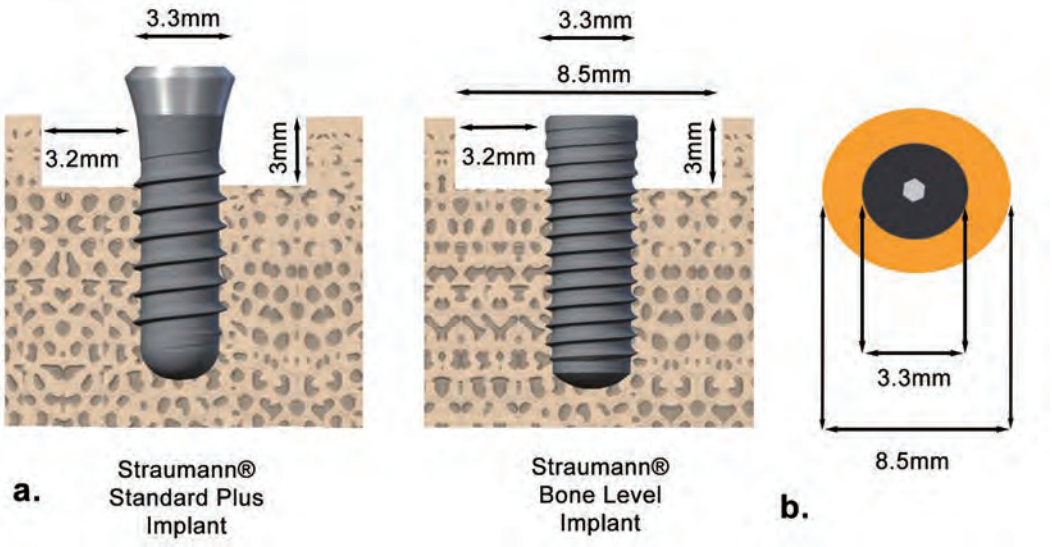

Figure 2. (a). Diagram of defect dimensions around (L) tissue-level and (R) bone-level implant. (b). Top-down view of defect with central implant.

Zealand) and plasticiser (Dibutyl phthalate 99%, Aldrich Sigma New Zealand). Each specimen was transferred into a glass jar with pre-set MMA III base and paper label, the jars filled with MMA III and placed in a water bath at RT for 4 weeks. Once set, the glass jars were broken and the resin-embedded specimens trimmed and polished prior to final sectioning. A Struers Accutom 10/100 precision table-top cut-off machine (Ballerup, Denmark) with a diamond cut-off wheel (MOD 13: 127 x 0.4 x 12.7 mm) was used to cut the resin-embedded specimens into sequential 750 µm thick-sections, which were again radiographed (Figure 3). before being mounted onto an opaque acrylic slide using cyanoacrylate glue and custom-designed press. The sections were ground and polished using a Tegra-Pol rotating grinding machine (Tegra-Pol, Struers, Ballerup, Denmark) and waterproof Silicon Carbide papers (grit sizes #500 to #4000). The final thickness of the slides was between 80 and 130 µm as determined using a digital micrometer (Digital Indicator, Mitutoyo, Japan). After superficial decalcification and etching with 20% ethanol followed by 0.1% formic acid in an ultrasonic bath for 5 minutes per step, sections were stained with one-part MacNeal's tetrachrome and two parts toluidine

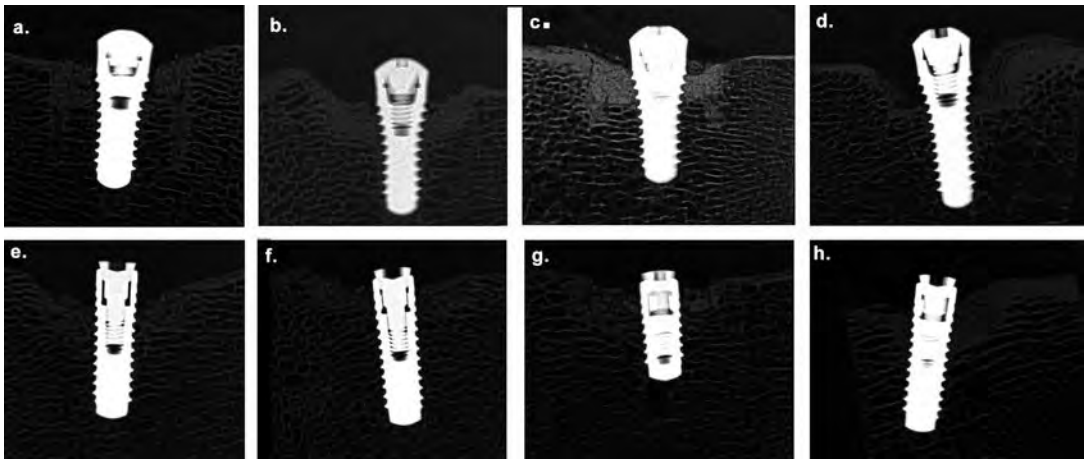

Figure 3. Representative radiographic images of 750 μm thick sections prior to mounting, grinding and polishing. (a). OMBAgNP after 8 weeks; (b). OMB after 8 weeks; (c). BioOss® after 8 weeks; (d). None after 8 weeks; (e). OMBAgNP after 12 weeks; (f). OMB after 12 weeks; (g). BioOss® after 12 weeks; (h). None after 12 weeks

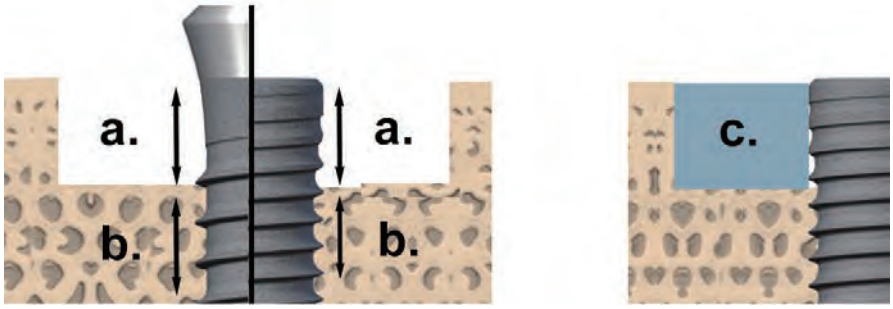

Figure 4. Diagram of measurements. (a) % Bone-implant contact (%BIC), Threads in graft. (b). % BIC, Threads apical to the grafted sites. (c). % Defect fill

blue solution for 5 minutes, then rinsed with distilled water and left to air dry (Kolonidis *et al* 2003 ; Duncan 2005).

Imaging and measurement

From each specimen, two adjacent slides representing the middle of the implant were selected.

High resolution images were acquired and digitised at 10x magnification using an inverted light microscope (Nikon Eclipse TiU series, Nikon Australia Pty Ltd) with an imaging system (IS capture, Tucsen Photonics Co. Ltd., China) linked to a laptop computer.

A computer-based image analysis system (Image J, National Institute of Health, USA) was used to measure the percentage of bone-to-implant contact (%BIC) adjacent to the portion of the implant lying within the grafted region, on each side of the specimen. Then, %BIC to the three threads within the trabecular bone lying immediately beneath the grafted region were measured, on each side of the implant (Figure 4a). Thus, for the N=2 sheep per time point, there were two histological specimens obtained for each of the four test conditions, with N= 12 measurements in total for the %BIC in the grafted regions, and N=12 for the un-grafted threads apical to this. The mean %BIC and standard deviation for each test condition at each time point was obtained from this data.

In addition, the area of the grafted region adjacent to each implant was measured and the percentage bone fill determined for each test condition and the two time periods (Figure 4b).

Statistics

Descriptive statistics (mean and standard deviation) were calculated for all variables using sheep as a statistical

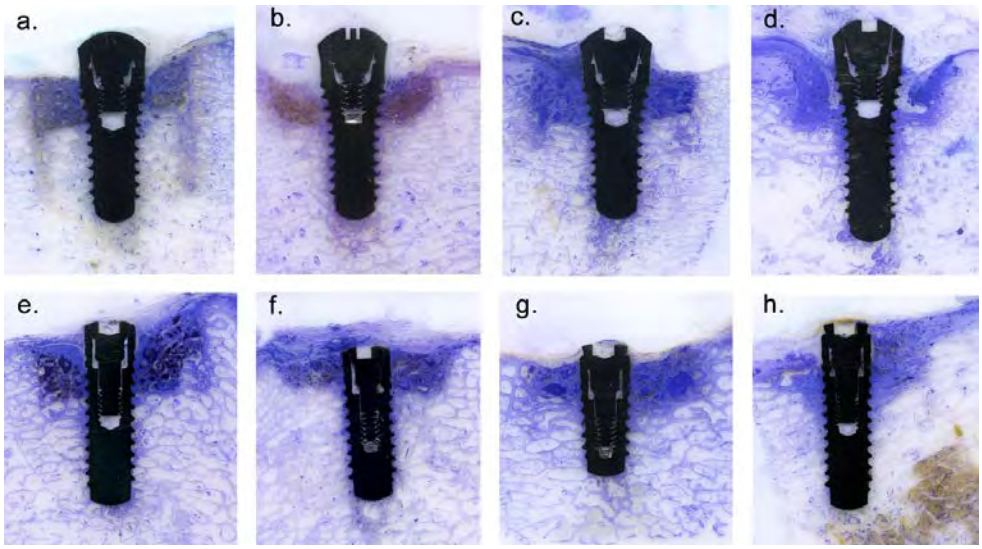

Figure 5. Scanned representative images of histology. (a). OMBAgNP after 8 weeks; (b). OMB after 8 weeks; (c). BioOss® after 8 weeks; (d). None after 8 weeks; (e). OMBAgNP after 12 weeks; (f). OMB after 12 weeks; (g). BioOss® after 12 weeks; (h). None after 12 weeks. MacNeils Tetrachrome and Toluidine blue stain.

unit. Due to the small sample size, data was not compared statistically between time points or between groups in this pilot study.

Results

All sites healed adequately without infection and without obvious effect on the animal's health; the average weight of the animals at surgery was 69 kg and at euthanasia, 73 kg.

Description

Histologically, in the three grafted groups (MB, OMBAgNP and BioOss), bone filled the grafted site and lay adjacent to the implant surface (Figure 5). Trabecular bone had extended from the implant osteotomy onto the implant surfaces below the grafted zone and in most specimens osteoconduction and condensation of bone supporting the implants was evident. The control, un-grafted defects clearly had less bone fill, although new bone filled the more inferior portion of the defects and had established bone-implant contact. In all of the three grafted groups (MB, MAAgNP and BioOss®), residual graft material was apparent, in all groups the residual material was surrounded by newly-formed bone that completely encapsulated the graft particles.

Table 2: Results for percent bone implant contact at 8 and 12 weeks; all groups combined including "None".

Healing	Threads in graft	Threads apical
period	%BIC (SD)	%BIC (SD)
8 week	52.1 [37.7]	83.9 [16.0]
12 week	61.3 [37.5]	87.2 [15.9]

Percentage Bone-implant contact

Overall, when %BIC in all the defect sites was combined and compared with the %BIC in the threads apical to the grafted sites, there was less %BIC in the grafted zone for all conditions at both time points; all measurements increased with longer healing periods. There was marked variability in the grafted zone measurements whereas the apical threaded regions were more consistent (Table 2).

The four different test conditions showed different responses. After 8 weeks, the lowest %BIC was found for the un-grafted ("None") control sites and this remained the case after 12 weeks although the %BIC appeared to increase over time. The poorest response for grafted sites was found with the OMBAgNP after 8 weeks, however this increased markedly after 12 weeks and became similar to BioOss®. The OMB graft had better %BIC than BioOss® at 8 weeks, however by 12 weeks these sites had poorer %BIC than all other graft materials and has less %BIC than the un-grafted controls ("None"). None of the grafting conditions appeared to influence the healing around the apical threads, which were

Table 3: Results for percent bone implant contact at 8 and 12 weeks for each test group.

	8 weeks		12 weeks	
Group	Threads in graft	Threads apical	Threads in graft	Threads apical
	%BIC (SD)	%BIC (SD)	%BIC (SD)	%BIC (SD)
OMBAgNP	43.6 [43.1]	84.6 [15.4]	64.6 [32.0]	78.5 [17.6]
BioOss®	61.1 [33.0]	84.3 [17.2]	78.6 [17.6]	95.0 [11.3]
OMB	66.3 [27.5]	86.1 [11.4]	44.9 [44.1]	82.6 [16.5]
None	37.3 [39.5]	80.8 [19.3]	56.9 [37.2]	92.6 [12.0]

Table 4: Results for percent bone implant contact for each test group, 8 and 12 weeks combined.

	8 and 12 weeks combined	
Group	Threads in graft	Threads apical
	%BIC (SD)	%BIC (SD)
OMBAgNP	54.1 [39.0]	81.5 [16.7]
BioOss	69.8 [32.0]	89.6 [15.4]
OMB	55.6 [37.9]	89.4 [14.2]
None	47.1 [39.2]	86.7 [17.0]

very similar at both time points for all implants.

When the data for the two time points were combined (N=4 sheep), the two test grafts (OMB and OMBAgNP) had similar %BIC and were better than un-grafted controls, but less than the BioOss®-grafted defects; however, the very large standard deviations mean that these differences may have little validity. All the apical threaded portions showed consistently good %BIC.

Percentage Defect fill

Comparison of the percentage fill of the defects adjacent to the implants found that OMBAgNP had the greatest defect fill after 8 weeks (88%), with little difference between BioOss® (81%) and OMB (79%). All grafted sites demonstrated better fill than the un-grafted control (63%). After 12 weeks, BioOss® appeared to show the greatest increase in bone fill (92%), but there was little difference between this and the OMBAgNP (84%) or OMBAgNP (80%). The un-grafted control sites also showed increased bone fill (77%) over time but this remained less than the grafted sites (Table 5).

Discussion

This pilot study examined the response of two test bovine bone xenograft (BBX) materials, optimised MoaBone® (OMB) and optimised MoaBone® with silver nanoparticles (OMBAgNP) and compared this with a widely-used well-characterised commercially-available bovine bone xenograft (BioOss®) and a control defect with no grafting material. All defects were covered with a commercially-available resorbable collagen membrane, thus we expected to see some regeneration of bone in the control defects. Two time points were examined, 8 weeks and 12 weeks, which would correspond with approximately 3 to 4 months healing in human patients, at which point it might be expected that implants with circumferential bone defects that had been placed using a submerged protocol would be re-exposed for the placement of healing abutments and subsequent restoration and occlusal loading.

These preliminary results suggest that both test BBX materials achieved equivalent healing with respect to bone fill when compared to the commercially-available BBX BioOss®, and all the grafting materials were superior to un-grafted control sites at both time points. All grafting materials were encapsulated in newly-formed vital bone, however the relative proportions of bone, residual grafting material and fibrous connective tissue has yet to be determined using more detailed histomorphometric analysis.

We also considered the effect of the grafting materials on the formation of new bone in intimate contact with the implant surfaces. None of the grafting materials achieved the same %BIC within the grafted region as was found for the more apical portions of the implant. There was quite significant variation for %BIC for each sample within the grafted zones; this was due to a progressive increase in %BIC as measurements moved from the most coronal portion to the most apical portion of the grafted zone. With respect to osseointegration within the grafted regions, OMB and BioOss® appeared equivalent after 8 weeks and superior to OMBAgNP, however this finding was reversed after 12 weeks. There was no evidence that either the test MoaBone® graft material itself nor the presence of AgNP impeded

Table 5. Percent defect fill after 8 and 12 weeks

	8 weeks	12 weeks
Group	Defect fill	Defect fill
	% (SD]	% (SD]
OMBAgNP	88.1 [5.1]	84.3 [1.7]
BioOss®	81.3 [6.5]	92.5 [2.4]
OMB	79.1 [2.3]	79.9 [2.9]
None	62.6 [8.4]	77.1 [2.0]

osseointegration within the grafted sites, however the poorer performance of the test materials when compared to BioOss® requires more detailed study using sufficient animals for valid statistical comparison. All the grafting materials resulted in greater %BIC than the non-grafted control sites. None of the materials affected osseointegration of the body of the implant apical to the grafted zone; in particular, the presence of AgNP had no obvious deleterious effect on the osseointegration of the implants.

A variety of technologies for coating silver nanoparticles onto titanium implants have been investigated *in* vitro for their antibacterial efficacy and effect on mammalian cells (Haugen *et al* 2022). However, there are only a few reports of the *in vivo* osseointegration response to dental implants augmented with AgNPs. Straumann SLA-surface implants with silver-ion impregnated surfaces were investigated in a dog mandibular intra-oral model by Qiao *et* al (2015). At 12 weeks, %BIC for the Ag-impregnated implants ranged from 69 to 73%, compared with 62 to 66% for the SLA-surface implants. In another study, blasted and acid-etched titanium implants coated with silver-doped polysiloxane were placed into pig calvarium for four months and demonstrated a statistically non-significant lower bone-implant contact (63%) compared with uncoated implants (77%) (Smeets *et al* 2017).

The implants used in our study came from the same manufacturer and were of consistent diameter, however there was some variation in implant length, surface (SLA or SLActive), material (some implants were Roxolid® and some were cp Titanium) and configuration (tissue-level or bone-level implants). Whilst these difference may have influenced results, the consistency seen in %BIC in the apical portion of the specimens suggests that this effect would have been minor. Nevertheless, for any subsequent quantitative study designed to determine differences between the response to the grafting materials, these variables would need to be controlled. Furthermore, these defects were created in a femur site and fully buried during healing; future testing should aim to expose the healing graft and implant sites to intra-oral infection, such as occurs in our large animal peri-implantitis and immediate implant models(Duncan *et al* 2018).

Conclusions

Both of the modified test bovine bone xenografts (OMB and OMBAgNP) showed broadly equivalent results when compared with a commercially-available BBX (BioOss®) and appeared superior to un-grafted controls. The addition of silver nanoparticles did not seem to impair bone healing. This pilot study supports the progression to larger-scale quantitative preclinical trials which should include testing in intra-oral animal models with infected sites.

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Enhancing Prognosis of Severely Compromised Tooth with Periodontal Regeneration

Ho King Lun, Dominic[1], Tse Yuen Kwan, Ingrid[2], Fok Melissa Rachel[1]

[1] Clinical Assistant Professor in Periodontology and Implant Dentistry Faculty of Dentistry, University of Hong Kong, Hong Kong, China SAR
[2] Senior Clinical Practitioner in Periodontology and Implant Dentistry Faculty of Dentistry, University of Hong Kong, Hong Kong, China SAR

The World Health Organization predicted that by 2030, 1 in 6 people will be aged 60 years or above. Population aging, accompanied by the declined edentulism prevalence with improved dental care, have led more elderly patients seek dental treatment for their compromised dentitions due to various dental conditions, such as periodontal disease, tooth substance loss or a combination of both (Al-Rafee 2020). The "extraction and replacement" strategy has been adopted to solve patients' dental problems over the past decades. However, with patients' surging demands and the perceived benefits for the preservation of their natural dentition, it is becoming more challenging to fulfil their expectations by such an approach.

The "extraction and replacement" strategy is a commonly adopted treatment modality utilizing fixed or removable prostheses to replace teeth with compromised prognosis. Dental implants for teeth replacement in complete edentulous and partially dentate patients have demonstrated high survival rates (Jung *et al* 2012 ; Pjetursson *et al* 2012). Hence, extracting a tooth with compromised periodontal prognosis appears to ease management in most clinical situations. The systematic review published by Jung and co-workers had evaluated 46 clinical studies and reported the survival rate of dental implants to be 97.2% (95%CI: 96.3-97.9%) and 95.2% (95%CI: 91.8-97.2%) after a mean observation period of 5 and 10 years respectively (Jung *et al* 2012). Nevertheless, it is well understood that extraction and replacement with dental implants is not an ultimate failproof solution for replacing teeth with compromised prognosis. It is not uncommon to encounter biological complications like soft tissue complications (7.1% (95% CI: 4.4-11.3%)), crestal bone loss (5.2% (95% CI: 3.1-86%)) and alveolar bone loss (Jung *et al* 2012). Technical complications, such as screw loosening (8.8% (95% CI: 5.1-15%)), loss of retention (4.1% (95% CI: 2.2-7.5%)) and veneering fracturing (3.5% (95% CI: 2.4%-5.2%)) are not to be overlooked (Jung *et al* 2012). A higher implant biological complication rate is also expected for periodontally compromised patients. A systematic review with 27 publications published by Sousa *et al*. revealed that implants installed in patients with treated-periodontitis demonstrated 79.22% to 100% survival rate versus 91.67% to 100% in non-periodontitis patients over a 1.2 – 16 years observation period (Sousa *et al* 2016). It was also reported that patients with treated periodontitis demonstrated more significant bone loss around dental implants and higher incidence of peri-implantitis than non-periodontitis patients in 5- and 10-years follow-up period (Sousa *et al* 2016).

Apart from those potential biological and technical complications, complexity of the implant surgery may also deter operators and patients from extracting and replacing a tooth with compromised periodontal prognosis with dental implant. The surgical placement of a dental implant is sometimes not as straightforward after extracting a periodontally compromised tooth, often requiring advanced augmentation procedures and maxillary sinus manipulation at molar areas (Fok *et al* 2020). Implant surgery is generally considered safe and predictable for most of the individuals. Schimmel *et. al*. reported that geriatric patients with polymorbidity, such as cardiovascular disease, Parkinson's disease, Diabetes Mellitus Type II, cognitive disease and even patients under low dose anti-resorptive therapy, could still benefit from the high survival rate of dental implants (Schimmel *et al* 2018). It is also critical that modifiable risk factors, such as diabetes mellitus and smoking, have to be controlled prior to implant surgery to prevent short- and long-term complications. Most of the medical conditions, such as increased bleeding tendency, are considered as relative risk factors which may only complicate the execution of implant surgery but not jeopardize implant survival. However, exceptions may include cancer patients who are under the influence of radiotherapy and patients who are undertaking high-dose anti-resorptive therapy. These patients may be

subject to an elevated risk of implant complications, such as osteonecrosis of the jaw. Tooth extraction and implant surgery in patients with altered bone metabolism may become undesirable to both the patient and clinician in this situation.

Deficiency in alveolar ridge volume may also favour the retention of a natural tooth over extraction and replacement with dental implant. The consensus report of the 15th European Workshop on Periodontology on Bone Regeneration concluded that bone augmentation procedure with various techniques is an effective procedure to overcome vertical and horizontal alveolar ridge defects as simultaneous or staged procedures (Jepsen *et al* 2019). A weighted mean complication rate was reported to be 16.9% (95% CI: 12.5-21) and it varies among different types of procedures (Urban *et al* 2019). Placement of dental implant in atrophic mandible may encroach the safety distance between the implant and the neurosensory nerve bundle. In extreme situations, some authors suggested Inferior Alveolar Nerve IAN lateralization / transposition of neurosensory bundle (Abayev and Juodzbalys 2015). Techniques to overcome the vertical deficiency includes guided bone regeneration, block graft technique and distraction osteogenesis. Vertical ridge augmentation is, however, highly technique sensitive. The weighted mean of vertical ridge augmentation with various techniques was reported by Elnayef *et. al.* to be 4.49 +/- 0.33 mm (95% CI: 3.85 to 5.14mm). Post-operative complications, such as neurosensory disturbance, wound infection, device-related complications, etc. may occur (Elnayef *et al* 2017). Among various post-operative complications, it is again not uncommon to encounter neurosensory disturbance after neurosensory bundle lateralization and transposition. Vetromilla *et al.* reported in a systematic review that , up to 95.9% and 58.9% of patients who received inferior alveolar nerve (IAN) lateralization and transposition experienced short-term neurosensory disturbance during the post-operative period respectively. Up to 3.4% and 22.1% of the patients, respectively, remained affected by neurosensory alteration at the end of the study. The repositioning of inferior alveolar nerve is, therefore, considered a high-risk dental implant-related surgery and more scientific evidence is required to prove its safety for clinical applications (Vetromilla *et al* 2014).

The advanced destruction of the dental alveolar ridge subsequent to periodontal inflammation, and the close proximity of the nearby vital anatomical structures to periodontal lesions (Fig 1) also impose elevated intra-operative risks, such as nerve injury. Extraction and inserting an implant may not necessarily simplify the clinical situation for both operators and patients as one may expect when compared with other treatment modalities to preserve the natural dentition.

Periodontal regeneration has been demonstrated to be a successful treatment modality to repair infra-bony periodontal defects. Its aim is not only to regenerate the lost periodontal apparatus but also to eliminate the infra-bony periodontal defects, to improve tooth prognosis and to facilitate long term periodontal maintenance of the offending tooth. The classic narrow, deep and 3-walled periodontal defects have been demonstrated to benefit from periodontal regenerative procedures with 1.1 to 1.3 mm more clinical attachment gain when compared to access flap periodontal surgery alone (Needleman *et al* 2002 ; Trombelli *et al* 2002). Different research groups had been testing the limit of the periodontal regenerative approach over the decades. Cortellini and Tonetti had demonstrated long-term success of periodontal regeneration with long-term stable outcomes on periodontally-involved teeth with advanced attachment loss in their retrospective investigation (Cortellini and Tonetti 2004). Teeth with advanced clinical attachment loss up to 10.7 +/- 2.4 mm and deep intra-bony defects up to 6.6 +/- 2.1 mm were treated with Guided Tissue Regeneration. The periodontal regeneration resulted in tooth survival of 96% following an observation period over 10 years. 92% of the cases demonstrated coronal displacement of the clinical attachment level in relation to their pre-treatment level even after 15 years of the operation.

Although promising long-term outcomes has been shown beyond doubt that periodontal regeneration can be a successful treatment modality even when performed on teeth with advanced attachment loss (Cortellini and Tonetti 2004 ; Nygaard-Ostby *et al* 2010 ; Pretzl *et al* 2009 ; Sculean *et al* 2008), one may argue that the benefit-cost ratio of "rescuing" a tooth with advanced periodontal attachment loss close to or beyond the tooth apex may not justify such regenerative approach when compared to the extraction and replacement strategy. For a tooth with such an advanced attachment loss, it may present with excessive mobility and the endodontic status may also be jeopardized leading to an endodontic-

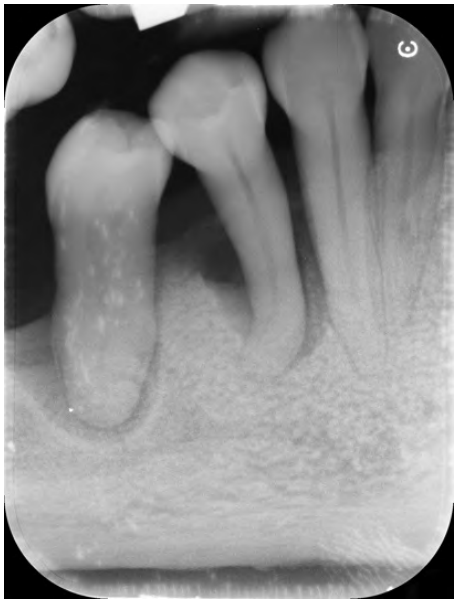

Figure 1. Periodontal lesion in close proximity to neurosensory bundle.

periodontal lesion. Management of hypermobility, occlusion and elective endodontic treatment may also be prescribed prior to periodontal surgical intervention in selected cases since mechanical instrumentation may disrupt the peri-apical blood supply to the pulpal tissue and cause post-operative endodontic complications. Adding the cost of potential endodontic treatment, the overall treatment fee for rescuing a tooth with advanced periodontal attachment loss is likely to surpass the costs of extraction and replacement with a dental implant.

A randomized clinical controlled trial published by Cortellini *et al.* had compared the medium-term outcome of periodontal regeneration approach with extraction and prosthetic replacement of teeth with severe attachment loss up to 5 years observation period (Cortellini *et al* 2011). Fifty patients suffering from severe periodontitis with at least one tooth with periodontal destruction up to or extending beyond tooth apex were recruited and were randomly assigned to either extraction and replacement with implant- or tooth-supported fixed prosthesis and periodontal regeneration to save the "hopeless periodontal prognosis" tooth. The replaced units (in extraction and replacement group) and the treated hopeless teeth (in periodontal regeneration group) had reached 100% and 92% survival after 5 years of observation period, respectively. Only 2 out of the 25 "hopeless teeth" in the periodontal regeneration group were extracted after the first-year survival due to inadequate improvement of the clinical situation. Considering over half of the studied teeth were graded into "poor" to "hopeless" categories according to McGuire & Nunn at baseline (McGuire 1991), the high survival rate of teeth in the regenerative group was very impressive and encouraging to individuals who would like to rescue periodontally compromised dentition. It was also interesting to look into the performance of the prosthesis and the natural teeth groups during the 5-year observation period. Both groups were performing very similarly in terms of the incidence of negative events defined by tooth loss, clinical attachment level loss, bone loss or probing depth increase >/=2mm.

In a 10-year follow-up study by Cortellini and co-workers, the test group which received periodontal regeneration only experienced 1 further tooth loss at the 8th year and still reached 88% survival after 10 years (Cortellini *et al* 2020). The complication-free survival time for periodontal regeneration group, and extraction and replacement group was 6.7-9.1 years and 7.3-9.1 years, respectively. The difference did not reach statistical significance. Cortellini *et al.* had further compared the two treatment approaches comprehensively by looking into the economic aspects and patient-reported outcomes. The periodontal regeneration approach costed higher than the extraction and replacement approach for the cost of recurrence in the short term, but they reached very similar levels in 10 years' time. However, the total cost of extraction and replacement group was at all-time higher than the periodontal regeneration group. It was also intriguing that patients who received periodontal regeneration achieved significantly greater improvement in OHIP-14 score when compared to patients who received the extraction and replacement approach. Although majority of the patients (88%) showed strong eagerness on preserving the severely compromised tooth at the beginning of the study, which might lead to some degree of sampling bias, these findings serve as an evidence to support that salvaging severely compromised teeth was not just solely a clinician's preference. The results also provided a very strong support to extend our understanding of the efficacy and predictability of periodontal regeneration on improving the prognosis of severely periodontally compromised teeth. With proven long-term favourable treatment outcomes and benefiting patients'

quality-of-life, periodontal regeneration has demonstrated itself to be an economically-justifiable alternative to tooth extraction in carefully-selected cases.

The publication by Cortellini and co-workers may give readers a hint on the key for success in periodontal regeneration on the severely periodontally compromised tooth (Cortellini *et al* 2011). Other than well-controlled patient, tooth and site level risk factors, meticulous plaque control and a high quality execution of the minimally invasive periodontal surgical technique, which can promote post-operative wound and blood clot stability, the presence of intra-bony periodontal defects with a clearly detectable bone crest at the adjacent tooth is one of key features for success in periodontal regeneration under this challenging condition.

Case 1

A 37-year old medically healthy non-smoking gentleman presented with a tooth 33 with an endodontic-periodontal lesion and severe attachment loss beyond the apex (Fig. 2a). Patient received orthonagthic surgery more than 10 years ago and received regular dental care at a private general dental clinic. The dentist noticed that the tooth 33 developed increasing hypermobility over the recent years and the patient was referred to an endodontist for determining further treatment due to radiolucency involving the apex. Tooth 33 presented with mobility degree 1 and increased probing depth for up to 12mm at the disto-buccal aspect with suppuration on probing. Tooth 33 received root surface debridement and composite splinting for stabilizing the tooth, and patient was referred back to the endodontist for completing the endodontic treatment. A 3-month period of healing was given and tooth 33 still presented with a persisting intra-bony periodontal defect extending up to the apex, excess endodontic obturation cement can be observe at the peri-apical region (Fig. 2b).

Tooth 33 was treated with air-polishing (EMS AIRFLOW® PLUS Powder, EMS, Switzerland) 2 weeks prior to the surgical intervention. On the day of the periodontal regeneration surgery, a 1-sided full thickness periosteal flap was raised with an entire papillary preservation approach (Aslan *et al* 2017). Root surface and the peri-apical area was exposed (Fig. 2c), the granulation tissue and residual cement was removed with surgical curette. Root surface was instrumented with an ultrasonic device (Airflow Prophylaxis Master, EMS, Switzerland) and treated with 2% EDTA for 2 minutes, followed by copious irrigation with normal saline. Enamel Matrix Protein Derivatives (Emdogain, Institut Straumann, Basel, Switzerland) and xenograft fillers

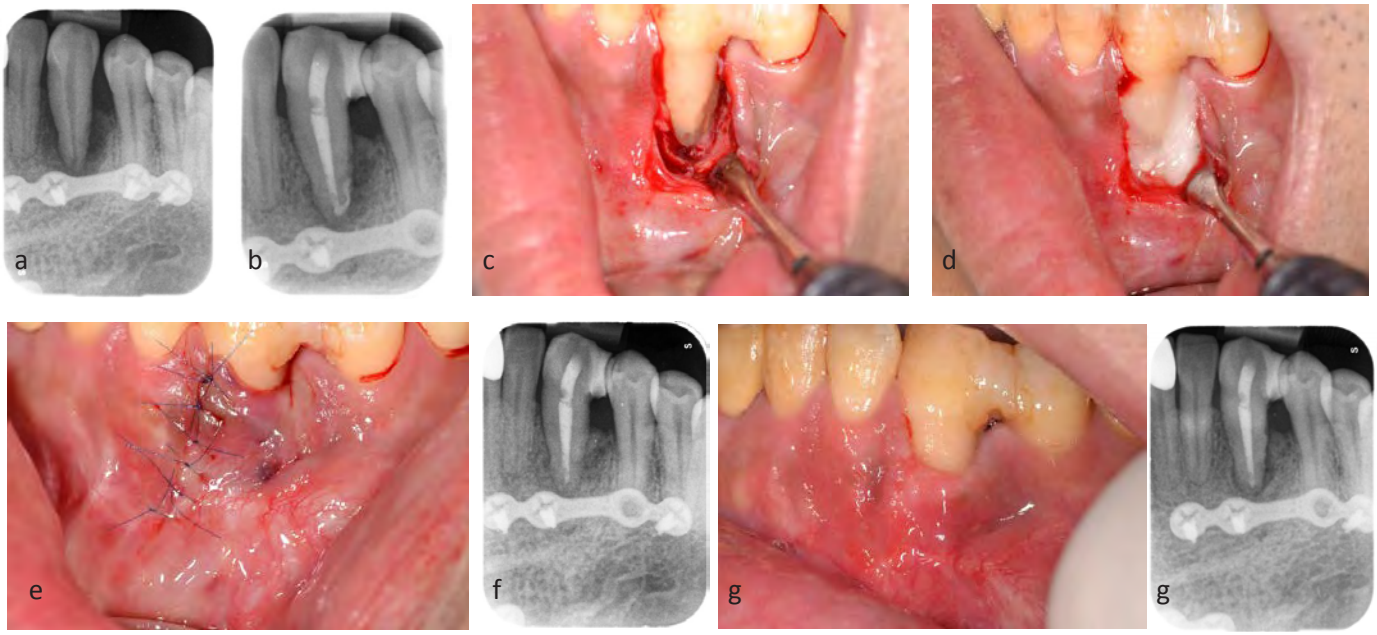

Figure 2. a.Baseline; b.3-month post endodontic treatment; c.Intra-operative photo d.Application of 2% EDTA and Enamel Matrix Derivatives; e.Immediate post-operation f.2-week post-operation; g.6-month post-operation

(BioOss Collagen, Geistlich Pharma AG, Wolhusen Geistlich) were applied to the intra-bony defect to support the flap upon closure (Fig. 2e). The periosteal flap was repositioned to the original position and stabilized with 7-0 Polyvinylidene Fluoride suture (Seralene® Blue 7-0, 0.5m DS-12) (Fig. 2f). Post-operation analgesics, systemic antibiotics and antiseptic mouthwash were prescribed for a week. Sutures were removed after a week of healing (Fig. 2g).

Case 2

A 42-year old medically healthy non-smoking gentlemen was referred from an Endodontist for assessment of the prognosis of an endodontically treated mandibular first molar. The tooth 46 presented with an existing provisional crown and received recent endodontic re-treatment. Periodontal examination revealed that there was a 10mm probing pocket depth at the mid-buccal aspect with a small soft tissue fistula at the buccal gingival margin, and the furcation involvement was up to Grade III sub-class C (Fig. 3a). Peri-apical radiograph showed an advanced radiolucency extending beyond the apex of the mesial root and also an isolated apical radiolucency at the distal root (Fig. 3b). Three months additional healing was given and the radiolucency at the distal root apex showed complete resolution. However, the periodontal defect at the mesial root remained (Fig. 3c). On the day of the operation, a one-sided flap was elevated from tooth 47 mesial to tooth 46 mesial aspect with a mesial vertical releasing incision. Full thickness flap was elevated until the buccal bone crest of the defect was exposed by just beyond 2mm. Degranulation of the defect was carried out with surgical curettes and root surface debridement was performed with ultrasonic instrument (Airflow Prophylaxis Master, EMS, Switzerland) and manual curettes (Fig. 3d). After thorough examination of the remaining tooth structure for exclusion of the root surface damage, followed by a well control of the bleeding, the root surface was treated with 2% EDTA for 2 minutes, copiously irrigated with physiological saline and modified with Enamel Matrix Protein Derivative (Emdogain, Institut Straumann, Basel, Switzerland). The space of the defect was filled with xenograft materials (BioOss Collagen, Geistlich Pharma AG, Wolhusen Geistlich) to support the flap stability. The flap was repositioned to the original position and stabilized with 5/0 ePTFE suture (Keydent PTFE dental suture, EP 1 (USP 5-0) 50 cm, needle DS-15) at primary closure position (Fig. 3e). Post-operation analgesics, antibiotics and antiseptic mouthwash were prescribed. The healing was uneventful and primary intention wound healing was maintained throughout the initial healing

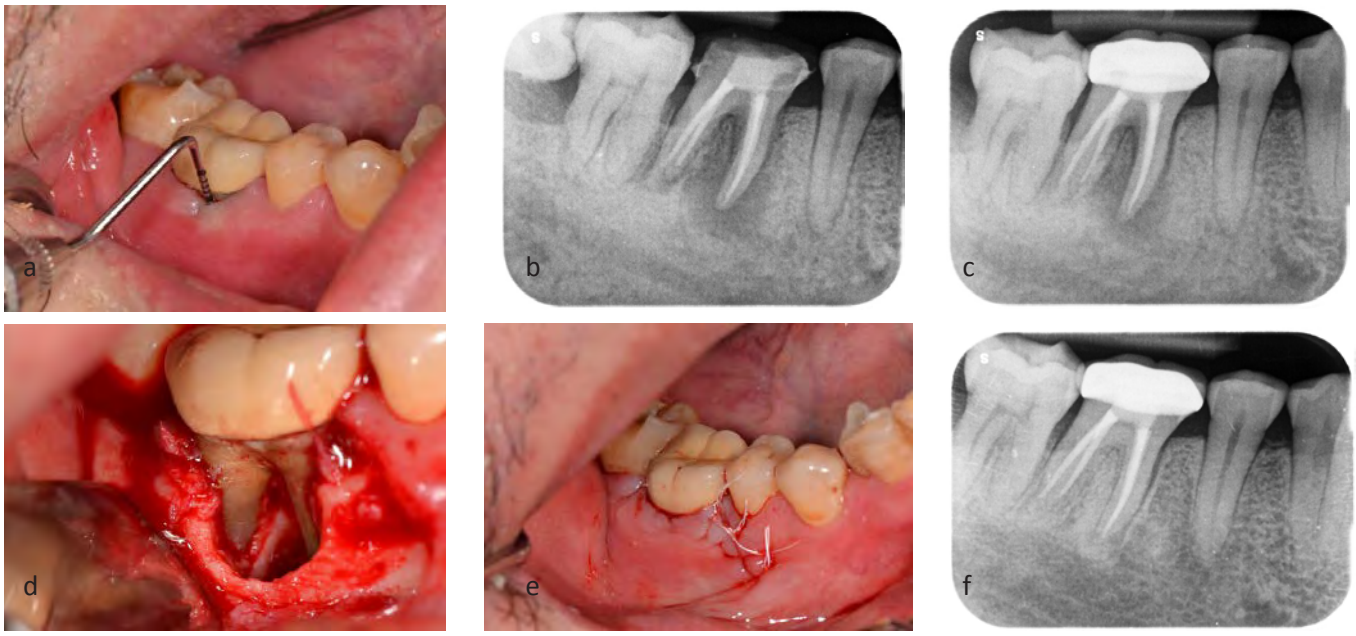

Figure 3. a. Pre-operation probing depth up to 10mm; b.Baseline radiograph; c.3-month post endodontic radiograph; d. Intra-operative photo; e. Closure of periosteal flap with ePTFE suture; f.20-month post-operation

period. Sutures were removed 1 week after the operation and the wound healing was supported with weekly review care until 1-month post-operation.

A 6-month post-operatiive peri-apical radiograph revealed a reduction in the size of the apical radiolucency (Fig. 3f). The tooth presented mid-buccal pocket depth of 2 mm and complete closure of furcation involvement. Tooth 46 had been followed up to 2.5 years, clinically free from any signs and symptoms, and it continued to show a gradual reduction in the size of the residual apical radiolucency at the mesial root tip.

Conclusion

Performing periodontal regeneration on a severely periodontally compromised tooth is a technique sensitive procedure and the treatment process may engage multi-disciplinary collaboration. With careful case selection, it could be a financially justifiable treatment alternative to the extraction and replacement approach for a severely periodontal compromised tooth. Despite our conventional wisdom about periodontal regeneration, the presence of intra-bony periodontal defects with a clearly detectable bone crest at the adjacent tooth seems to be one of the key clinical predictors for success in performing periodontal regeneration to rescue a tooth with periodontal attachment loss extending beyond the root apex. The patient's willingness to treat, strategic value of the tooth and the possibility in hypermobility management should also be carefully evaluated when deciding the treatment approach for a tooth with advanced periodontal attachment loss.

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Alveolar Ridge Preservation Following Extraction of Molars with Severe Periodontitis

Wenjie Hu, Yiping Wei, Tao Xu, Liping Zhao

Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China

Introduction

Dental implants have become a widely accepted treatment option to replace missing teeth. The clinical success of osseointegrated implants has been thoroughly documented (van Velzen *et al* 2015). Ideal functional and esthetic prosthetic rehabilitation following implant therapy is possible when there is availability of sufficient alveolar bone volume and favorable architecture of the residual alveolar ridge.

Periodontal disease, which causes destruction of the ligament and alveolar bone supporting the teeth, is the main cause of tooth loss in adults (Frencken *et al* 2017). Molars play an important role in oral function, while they present a challenge for periodontal treatment, and show a higher risk for tooth loss due to their anatomy and position (Dannewitz *et al* 2016 ; Baumer *et al* 2011). A systematic review showed that a certain amount of alveolar bone resorption occurred after natural healing, and more bone loss could be expected in molar sites (Couso-Queiruga *et al* 2021). Alveolar ridge preservation was effective in reducing the amount of ridge resorption following tooth extraction (Tonetti *et al* 2019 ; Horvath *et al* 2013 ; MacBeth *et al* 2017). The indications for alveolar ridge preservation procedures have widening from anterior intact extraction socket to the posterior region and damaged extraction sockets (Zhao *et al* 2018 ; Ben Amara *et al* 2021 ; Kim *et al* 2021 ; Lee *et al* 2021). However, little evidence is available regarding the benefit of ridge preservation at periodontally compromised molar extraction sockets.

We investigated alveolar bone changes, treatment modality alterations, and implant- related outcomes following ridge preservation on molar extraction sockets with severe periodontitis, compared to natural healing (Zhao *et al* 2018 ; Wei *et al* 2021 ; Zhao *et al* 2022 ; Wei *et al* 2022).

Ridge alterations following molar removal

The alveolar process is a tooth-dependent bone tissue that develops in conjunction with the eruption of a tooth (Marks & Schroeder, 1996). After tooth extraction, alveolar processes exhibited atrophic changes along with a reduction in vertical height and a decrease in width of the residual ridge (Araujo and Lindhe 2005 ; Chappuis *et al* 2013 ; Pietrokovski *et al* 2007). Schropp et al. investigated contour changes of the alveolar processes of posterior extraction sites by measuring stone casts and discovered a reduction in width of the residual alveolar ridge of up to 50% (5.0-7.0 mm) during a 12-month healing period, of which two-thirds of the reduction occurred within the first 3 months of healing (Schropp *et al* 2003).

Numerous studies have investigated alterations in alveolar process dimensions after tooth extraction, often with results presented as qualitative and quantitative changes at extraction sites free of infection (non-inflammatory sockets), where teeth were removed due to root fracture or caries (Araujo *et al* 2015 ; Walker *et al* 2017). Healing dynamics in periodontally compromised extraction sockets are associated with slower healing and cortication (Ahn and Shin 2008 ; Bertl *et al* 2018). Severe loss of alveolar bone height and width can occur following removal of molars with severe periodontitis. We have confirmed that the molar extraction sockets with severe periodontitis do resorb and change in various sites, especially at the buccal wall of the socket (Zhao *et al* 2019). This dimension shrinkage may affect the subsequent implant procedure, resulting in ridge deficiencies that can adversely impact long-term implant stability or aesthetics, necessitating additional reconstructive surgery.

Alveolar ridge preservation at molar extraction sites

Alveolar ridge preservation is defined as any procedure that takes place immediately after tooth extraction to preserve or increase ridge volume within or beyond the skeletal envelope that exists at that time (Hammerle *et al* 2012). Ridge preservation may reduce the need for further bone augmentation during implant placement, may reduce sinus pneumatization, and may increase the possibility of inserting implants without the need for a sinus augmentation procedure

in the posterior maxilla (Rasperini *et al* 2010 ; Cha *et al* 2019 ; Park *et al* 2020). Furthermore, the augmentation procedure for severely resorbed alveolar sockets provides better results in terms of bone regeneration when compared with traditional GBR procedures performed at the time of implant placement in untreated sites (Sisti *et al* 2012).

Reconstruction of alveolar ridge volume in molar extraction sockets affected by severe periodontitis presents clinical challenges. Kim et al. revealed that ridge augmentation in periodontally compromised extraction sockets (including anterior and posterior teeth) was safe, with an overall safety rate of 99.4% (Kim *et al* 2017). We evaluated and compared the dimensional alterations of soft and hard tissue in periodontally compromised molar sites that received a ridge preservation procedure to those left to heal spontaneously without any other intervention.

Surgical protocol

Patients were assessed by periapical radiographs, clinical photographs, and clinical periodontal examination to confirm the unsalvageable nature of the tooth to be extracted. At least 7 days prior to the surgery procedure, all patients underwent scaling, root planing, oral hygiene instructions, and any necessary supportive periodontal treatment to provide a more favorable oral environment for wound healing.

Following the administration of local anesthesia, an internal bevel incision to the bone crest was performed approximately 0.5-1 mm below the buccal and the lingual free gingival margin of the tooth to remove the inner wall of the periodontal pocket without flap elevation. The ailing tooth was extracted atraumatically using periotomes and extraction forceps, and if required, roots were separated within the socket by using diamond fissure burs in order to avoid unnecessary trauma to the alveolar bone walls. Sockets were carefully examined and meticulously debrided with surgical curettes to remove all granulation tissue, and then were irrigated with sterile saline solution and curetted to stimulate fresh bleeding from the osseous base of the alveolar

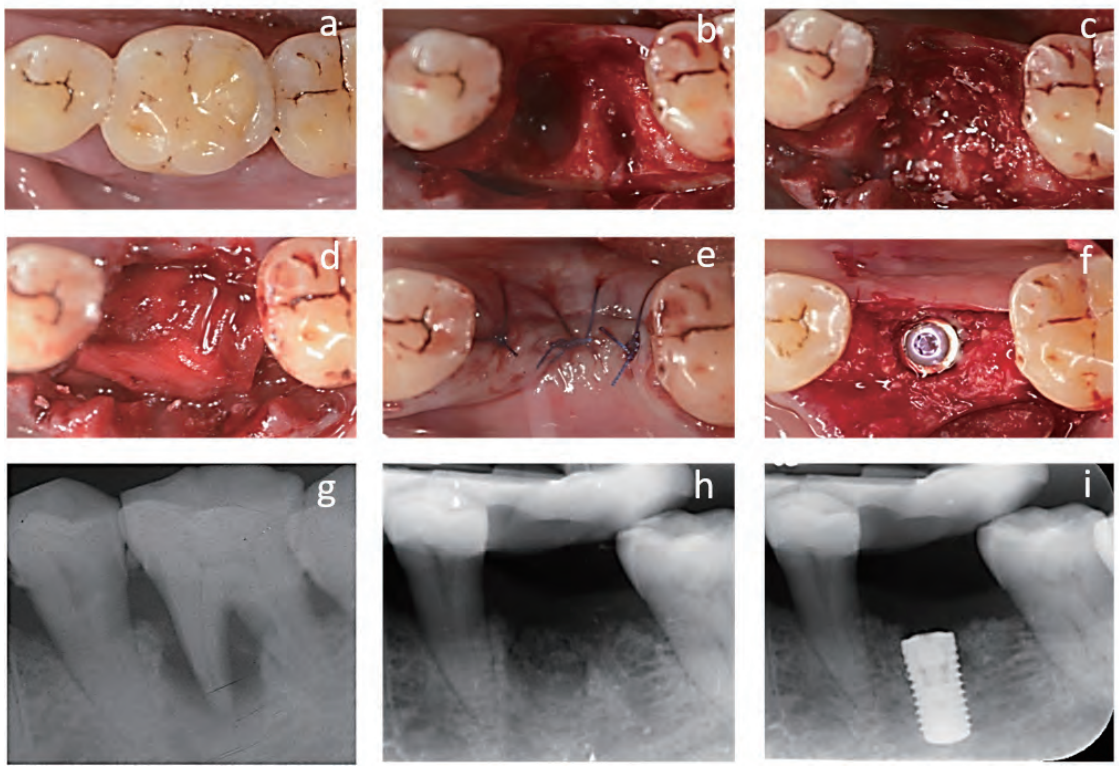

Figure 1 The primary healing intention group: (a) pre-extraction clinical aspect, (b) severe buccal bone defect after atraumatic extraction and vertical releasing incision in the buccal aspect, (c) socket grafted with Bio-Oss, (d) covered with Bio-Gide, (e) cross-mattress and interrupted sutures for primary wound closure, (f) flap elevation and implant placement following 6 months of healing; periapical radiographic images taken (g) before tooth extraction, (h) immediately after ridge preservation/augmentation, and (i) immediately after implant placement.

ridge.

We carried out two kinds of surgical procedures, ridge preservation with or without primary wound closure. The primary healing intention group received a full thickness mucoperiosteal flap procedure with two vertical releasing incisions beyond the mucogingival junction in the mesial and distal aspects of the sockets and extending to the mucosal vestibular groove at the buccal side. Sockets were filled with xenograft (Bio-Oss; Geistlich Pharma AG, Wolhusen, Switzerland) and the graft was placed loosely without condensation, which consists of small granules of particle size 0.25-1 mm. The graft was hydrated with sterilized saline up to the crest of the socket, winthin the confines of the ridge to avoid placing excessive graft particles. An absorbable collagen membrane (Bio-Gide; Geistlich Pharma AG, Wolhusen, Switzerland) was trimmed and applied to completely cover the socket with 2–3 mm extending over the alveolar bone crest. Subsequently, the buccal flap was advanced coronally to allow maximum primary soft tissue closure (Figure 1) (Zhao *et al* 2018).

In the secondary healing intention group, tunneling full-thickness flaps were elevated buccally and lingually for exposure of 2–3 mm of the alveolar bone crest of the socket. After sockets were filled with Bio-Oss and covered by Bio-Gide, the extraction site was then covered with medical collagen sponge (Wuxi BIOT), not requiring primary soft tissue closure (Figure 2) (Wei *et al* 2021).

Effects of alveolar ridge preservation at molar extraction sites

We reported that both ridge preservation techniques were effective in minimizing ridge resorption after tooth extraction. The micro-flap technique gave positive outcome in terms of the keratinized gingival width than that of the flap technique. Moreover, ridge preservation on maxillary molar extraction sockets with severe periodontitis maintained the vertical bone height more efficiently and resulted in less need for sinus augmentation procedures at 6 months compared to

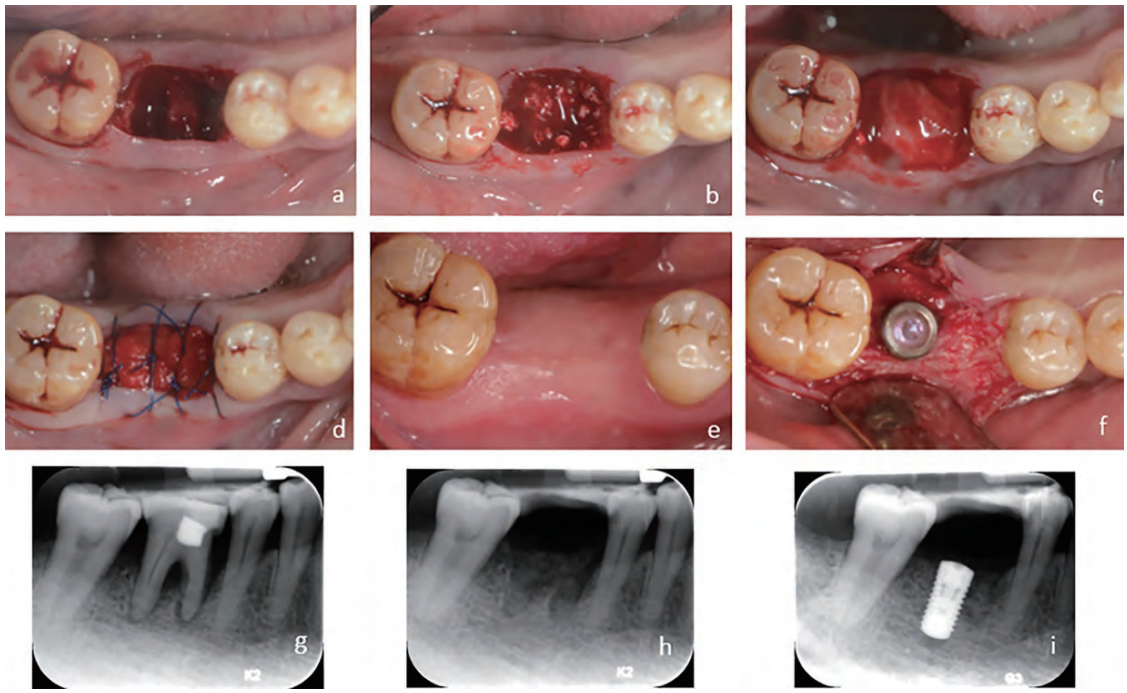

Figure 2 The secondary healing intention group a) Severe buccal bone defect after minimally invasive surgical extraction of the lower right first molar. b) Socket grafted with DBBM. c) Collagen membrane covered. d) Collagen sponge inserted to the upper area and cross-mattress sutures. e) Maturation of the soft tissues after a 6-month healing period. f) Implant placed in the preserved socket in a prosthetically guided position. g) Peri-apical radiograph of the tooth before extraction. h) Peri-apical radiograph of the grafted socket immediately after ridge preservation. i) Periapical radiograph immediately after implant placement.

natural healing (Wei *et al* 2022).

Implant-related outcomes following alveolar ridge preservation at molar extraction sites

Although numerous investigations have been done to reiterate the positive results of ridge preservation, data regarding the long-term prognosis of implants placed in ridge-preserved and naturally healed extraction sockets of periodontally compromised molars were still scarce. At the 1-year observation phase, the success rate of implants placed in augmented sockets ranged between 93% to 100% according to the definitions of success criteria; the success rates of implants placed in ridge-preserved sites and naturally healed sockets were equivalent (Apostolopoulos and Darby 2017 ; Patel *et al* 2013). We found that implants placed at ridge- preserved and naturally healed sockets of periodontally compromised molar demonstrated comparable outcomes with regard to survival and success rates, peri-implant parameters, and marginal bone levels at a 3-year post-loading evaluation (Zhao *et al* 2022).

Conclusion

Ridge preservation at periodontally compromised molar extraction sites can compensate for ridge width and buccal bone resorption that occurs with natural healing alone. Implants placed into periodontally compromised molar-extracted sites after ridge augmentation resulted in comparable outcomes to implant placement at naturally healed sites after 3-year functional loading.

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Horizontal Ridge Deficiency: Restorative Challenge in Anterior Maxilla

S Pradhan, S Sah, A Karanjit, S Adhikari

Department of Dental Surgery, Bir Hospital, Kathmandu, Nepal
National Academy of Medical Sciences, Kathmandu, Nepal

Abstract: Adequate amount of alveolar bone is critical for achieving functional prosthetic rehabilitation with adequate aesthetics. Implant placement in the anterior maxilla with deficient bone volume may be a difficult task to achieve a functional and esthetically pleasing outcome. Many procedures have been introduced to increase the amount of bone volume in sites chosen for dental implant placement. In addition to autogenous grafts being the golden standard of augmentation, various bone substitutes have been used with promising results. The main rationale in guided bone regeneration techniques is the creation of space for matrix producing cells if significant volumes of bone are to be achieved. This review highlights different techniques of correcting horizontal ridge deficiency with associated possible complications.

Introduction

Dental implants have become highly predictable treatments to rehabilitate, partially or fully, edentulous patients, with resulting cumulative survival rates ranging between 89.5% and 92.7% after 10–27 years of function (Balshi *et al* 2015 ; Chappuis *et al* 2013).

However, the bone resorptive changes occurring in the alveolar process after tooth extraction(Schropp *et al* 2003) or pathologic bone loss due to periodontitis, trauma or infection frequently leads to the lack of sufficient bone volume for placing implants in the ideal prosthetic position, which is a critical factor to attain the appropriate function and aesthetics of the implant- supported restorations. Due to the loss of teeth, the alveolar bone remodels and decreases in dimensions at varying rates and degrees(Schropp *et al* 2003). Research showed that, during the 6-month to 2-year post extraction period, there was a sharp reduction in the residual ridge volume(Tallgren 1969 ; Weinmann 1955). Regarding the bone changes after extraction, it has been demonstrated that the width of the alveolar ridge was reduced by 50% during the observation period. Approximately, two thirds of this reduction occurred within the first 3 months after tooth extraction. It demonstrated a greater reduction of the alveolar process in width than height, with a height reduction of 2.0 to 4.5 mm(Lang *et al* 2021).

The anterior maxilla is the most challenging area because of patients' esthetic demands and unfavorable pre-existing anatomy. Bone deficiency in the anterior maxilla prevents primary implant stability or results in an inadequate implant position with compromised esthetics or function(Dong *et al* 1999). Nowadays, patient awareness, particularly regarding time and esthetics, is high. Many patients have expectations of a short treatment time with perfect results, posing a significant challenge to both the clinicians and dental technicians. Whether a low or high smile line is present, many patients consider their maxillary anterior teeth to be one of their most important esthetic facial features(Henson *et al* 2011).

In addition, many patients also present with tissue deficiencies in the anterior maxilla because of traumatic tooth loss, periodontal or endodontic disease. Malformation and tumors are less frequently the cause of tissue loss in the anterior maxilla. Tissue deficiencies may include deficits of soft tissue (gingiva/alveolar mucosa) and/or hard tissue (alveolar bone). Bone deficiencies of the alveolar process may be categorized as vertical or horizontal deficiencies or combinations. Hard and/or soft tissue defects may lead to functional, structural or esthetic compromises in the final prosthesis(Wang and Al-Shammari 2002). Moreover, both removable or fixed implant-supported prosthesis is a challenge in the anterior maxillary region. When every step and sequence of treatment is properly executed, successful implant rehabilitation of the maxilla is one of the most satisfying procedures for both the restorative dentist and the patient(Bosse and Taylor 1998).

Various bone regenerative techniques have been proposed and evaluated to reconstruct deficient alveolar ridges to facilitate dental implant placement(Donos *et al* 2008 ; Rocchietta *et al* 2008). Depending on the morphology of the bone defect(Seibert 1983), these regenerative interventions

may have main objectives, such as horizontal, vertical or combined bone augmentation. Depending whether the implant could be placed with primary stability in the prosthetically driven position, these regenerative interventions could be simultaneous to the regenerative procedure or be with staged approach after implant placement(Benic and Hämmerle 2014 ; Kuchler and von Arx 2014 ; Merli *et al* 2016). It must be placed in a biologically acceptable and prosthetically driven position for a dental implant to function optimally. Hence, the three-dimensional positioning of the dental implant is crucial to a successful treatment outcome. The clinician is often challenged with a situation in which the remaining bone support is insufficient to place the dental implant in an ideal position. In addition, the soft tissue contour is often deficient in providing an esthetically pleasing treatment outcome(Schropp *et al* 2003).

Seibert introduced his widely used classification of ridge defects in 1983(Seibert 1983). As per this classification, Class I is buccolingual loss of tissue with normal apico-coronal ridge height, Class II is apico-coronal loss of tissue with normal buccolingual ridge width and Class III is combination-type defects (loss of both height and width). A new classification is a modification of Seibert's classification that attempts to address some of its limitations. The three broad categories are still present, with the use of simpler terminology, referring to Class I, II, and III defects as horizontal (H), vertical (V), and combination (C) defects, respectively.

In this classification, each category is further subdivided into small (s, ≤ 3 mm), medium (m, 4 to 6 mm), and large (l, ≥ 7 mm) subcategories. Both soft and hard tissue defects are considered in this classification scheme, with treatment options suggested based on the type and size of the defect and the planned restorative treatment plan.

Techniques to increase alveolar ridge width

Adequate horizontal ridge is prerequisite for prosthetically driven implant placement. Many, if not the most, cases in the anterior maxilla require horizontal ridge augmentation due to partial or complete loss of the facial bone plate following tooth extraction or tooth loss. It is often difficult to choose the most suitable treatment modality. To validate this decision-making process (Fu and Wang 2011) proposed 'The decision tree' which stems from the 3-dimensional buccolingual bone width available at the site of implant placement. Techniques are advised after considering factors such as the tissue thickness, arch position and availability of autogenous bone.

Sandwich bone augmentation (SBA) technique(Wang *et al* 2004 ; Fu and Wang 2012)

Sandwich bone augmentation (SBA) technique is a unique form of guided bone regeneration that can be used simultaneously with implant placement(Buser *et al* 1990).

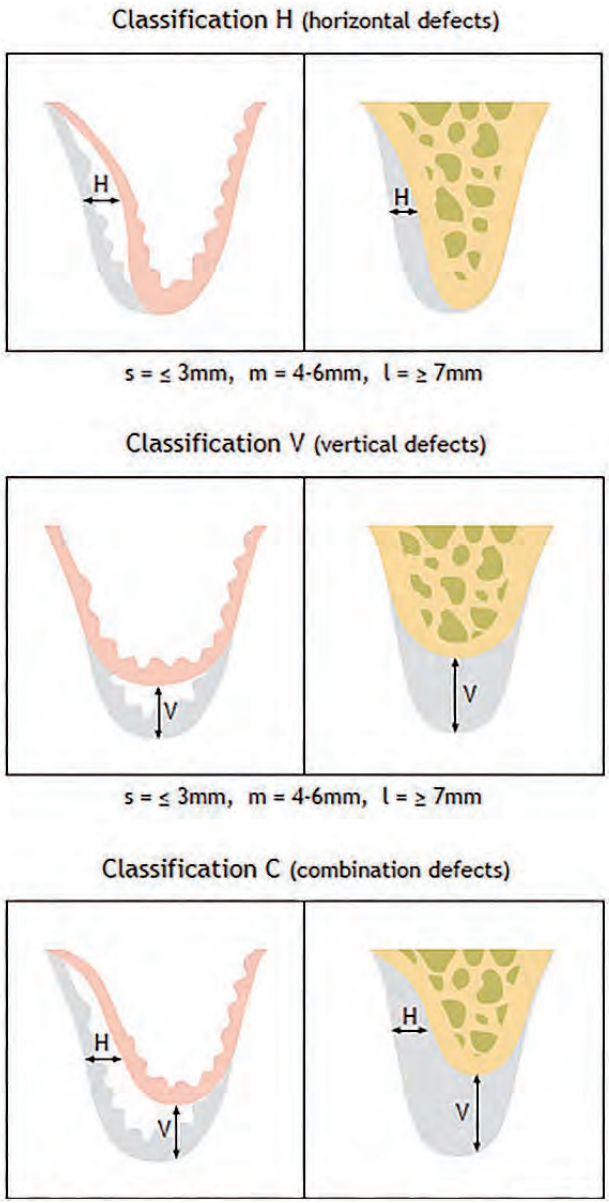

Figure 1: HVC ridge classification. Sub classification: small (s) ≤ 3 mm; medium (m) 4–6 mm; large (l) ≥ 7 mm(Wang and Al-Shammari 2002).

The main component of SBA technique is autogenous bone chips or a fast-resorbing particulate cancellous allograft, which constitutes the first layer and is applied immediately against the implant surface. A second layer of slow-resorbing particulate cortical allograft is placed before an absorbable membrane is used to cover the site. To ensure that the space needed for augmentation is created or maintained, bovine hydroxyapatite is layered (outer layer) over the grafted area. It is covered up to 2-3 mm (buccolingual direction) beyond the adjacent bone level. In addition, to avoid the invasion of soft tissue cells into the layered graft materials, the use of a barrier membrane is recommended. SBA is indicated in horizontal alveolar ridge defect in combination with immediate implant placement with predictable primary stability. The main advantages of SBA are reduced treatment time, elimination of a second surgical procedure, cost-effective and positive treatment outcomes for the patient.

There are some factors that need to be considered for the success of SBA Technique(Buser *et al* 1999)

1. Primary implant stability – It must be achieved prior to bone augmentation because mobile implants (micromovements of >100μm) often heal with fibrous encapsulation; thereby, compromising osseointegration.

2. Primary wound coverage with passive tension – A sealed environment would eliminate the negative influence of oral microbial flora and promote uneventful healing.

There are some limitations of SBA which includes technical difficulty associated with achieving primary stability, membrane exposure and inability of the resorbable membrane to maintain space.

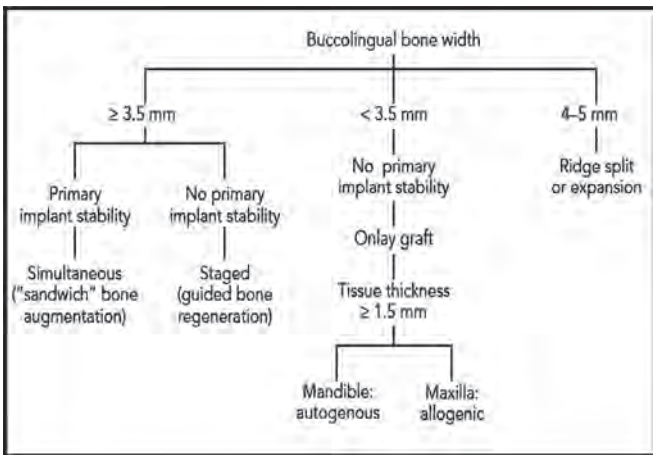

Figure 2: Decision tree for predictable horizontal ridge augmentation(Deepika-Penmetsa *et al* 2017)

Guided bone regeneration (GBR)/Staged approach

The American Academy of Periodontology (2001) defined GBR as procedures attempting to regenerate or augment bone for proper dental implant placement. GBR demonstrated predictable bone gain through PASS principle which includes primary wound closure, promoting angiogenesis, maintaining space for regeneration and obtaining primary implant and blood clot stability(Wang and Boyapati 2006). GBR is used as an alternative to simultaneous approach in treating dehiscence or fenestration defects. GBR and GTR are based on the same principles that barrier membranes are used for space maintenance over a defect, promoting the ingrowth of osteogenic cells and preventing migration of undesired cells from the overlying soft tissues into the wound.

A staged approach is preferred where autogenous bone grafts, either in blocks or particulate form, are firmly secured onto the ridge and a barrier membrane is placed. Primary closure of the wound site is attained, and the site is left to heal for 4 - 6 months before implant placement(McAllister and Haghighat 2007 ; Mellonig and Nevins 1995). The advantages of GBR approach includes space provision over a horizontal defect, promoting the in-growth of osteogenic cells as well as preventing migration of undesired cells from the overlying soft tissue.

Onlay graft (Block graft)

Onlay bone grafts are used for external augmentation of horizontal (veneer graft), or vertical alveolar deficiencies as well as combined defects (saddle graft). Autogenous bone grafts may be derived from intraoral or extraoral donor sites. Intra-oral sources for block grafts are symphysis, body and ramus of the mandible. Among them, ramus has been the preferred site as local consequences of graft harvest are less. Intraoral graft site is preferred when augmenting smaller defects. Allogenic bone may also be used as an onlay graft. Indications of onlay graft includes(Waasdorp and Reynolds 2010 ; Prasad *et al* 2014)

1. In situations where primary stability cannot be achieved in residual ridge width <3.5mm.

2. When bone harvested from the symphysis – can be used for predictable bone augmentation up to 6 mm in horizontal

and vertical dimensions.

3. When bone harvested from ramus – can be used for horizontal or vertical augmentation of 3 to 4 mm.

Advantages of onlay graft techniques are(Toscano *et al* 2010) large volume of cortico-cancellous block graft that can be harvested and carved into various shapes, provide an inherent ability to support the soft tissue, enhance revascularization of the cancellous portion, and provide mechanical support and rigidity of the cortical portion. However, onlay graft techniques are associated with some drawbacks(Waasdorp and Reynolds 2010) which includes;

1. Temporary paresthesia when harvested from chin.
2. Unpredictable graft resorption.
3. Higher risk of wound dehiscence and osseointegration failure.
4. Total graft loss.
5. Lower values of bone-to-implant contact and compromised implant position, thereby making the one-step procedure undesirable from a prosthetic viewpoint.

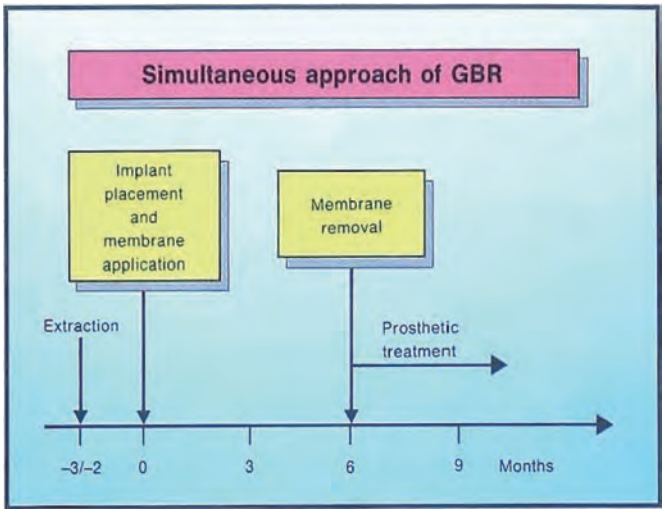

Figure 3: Simultaneous approach (Deepika-Penmetsa *et al* 2017)

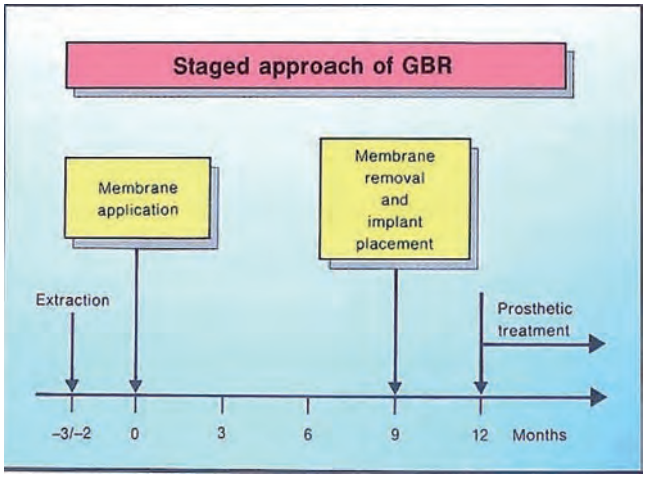

Figure 4: Staged approach (Deepika-Penmetsa *et al* 2017)

Ridge split or expansion technique(Scipioni *et al* 1994 ; Tolstunov and Hicke 2013 ; Bassetti *et al* 2016)

Ridge split with bone expansion is a technique of manipulation of bone to form receptor site for implant without removing any bone form the implant site. Maxillary bone has inherent quality of flexibility due to greater amount of cancellous bone which can be molded to desire location by using scalpel, chisels or osteotomes. An alveolar ridge demonstrating a narrow width (<5 mm in many cases) and grossly adequate alveolar height is the most common situation for the ridge split or expansion technique. The wider the cancellous bone layer (the layer where the split is done), the easier it would be to accomplish the ridge split procedure.

Conclusion

The major limitation of horizontally challenged ridge is the technical difficulty associated with achieving primary implant stability and prosthetically driven implant placement. Simultaneous implant placement with the GBR predictably regenerate bone on implant buccal dehiscence defects in horizontal deficiency. There are different techniques that allow the reconstruction of lost hard and soft tissue contours as well as implant placement in positions to permit prosthetic restorations that are optimal from functional and esthetic points of view.

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Detection of Sub-Gingival Calculus

BTK Tan

Private Practice, Singapore
National University of Singapore

Introduction

The ability to reliably detect sub-gingival calculus is essential for effective debridement of periodontal pockets. It also helps the clinician know when to stop debridement so that cementum is not unnecessarily removed.

The conventional method of detecting sub-gingival calculus is tactile examination using a periodontal probe. This is however difficult for both novice and experienced clinicians and there is limited reproducibility of the results between various operators(Pippin and Feil 1992).

Over the last 20 years, researchers have attempted to come up with novel methods of detecting sub-gingival calculus that are more effective and reproducible.

They include:

A surface recognition device that discriminates dental calculus and tooth surfaces by mathematical analysis of reflected ultrasound waves(Kocher *et al* 2000).

Induced fluorescence emission of dental calculus using a 655-nm-wavelength laser-based technology(Folwaczny *et al* 2004).

Differentiating light reflected from tooth surfaces illuminated with a light emitting diode (LED) with a wavelength of 635 nm(Krause *et al* 2005).

Periodontal endoscope(Geisinger *et al* 2007)

None of the above commercially available methods have been taken off. Manual detection using the periodontal probe remains the common method for clinicians to detect sub-gingival calculus today.

To the author's best knowledge, there has been no detailed description of how to use the periodontal probe to detect sub-gingival calculus in the literature. This paper gives such a description.

Probes commonly used to detect sub-gingival dental calculus

Whilst the conventional method for detecting subgingival calculus is tactile examination using a periodontal probe(Pippin and Feil 1992 ; Sherman *et al* 1990), the Old Dominion University (ODU) 11/12 explorer and sharp caries explorer are also commonly used.

The ODU 11/12 explorer is a double end explorer with angles similar to the Gracey 11/12. In *in vitro* studies, it has been found that the ODU 11/12 explorer was more effective in detecting sub-gingival calculus 0.5 to 1 mm sub-gingival on a typodont model than the WHO probe(Rams and Manos 2021) as well as Differential Reflectometry(Rams *et al* 2017). It is inserted into the periodontal pocket in the same manner as one would use the Gracey's curette.

The tip of the ODU is 3mm long and has a very sharp point. One must be careful when inserting this instrument in the manner as one would use a Gracey's curette into the buccal and lingual/palatal pockets. The sharp tip is likely to lacerate the soft tissue. This same risk applies when one inserts this sharp instrument into a deep pocket.

The ODU 11/12 explorer is therefore a useful instrument to check for calculus 0.5 to 1mm sub-gingivally at the inter-dental areas, but not ideal for use at the line angles, as well as on the buccal and lingual surfaces of roots.

The sharp caries explorer probe is also commonly used. There is an assumption that the sharp tip makes it more sensitive to feel for surface roughness. The clinician can easily check if this assumption is true by comparing for himself/herself the feel of a sharp probe vs that of a periodontal probe on either a tooth or on any convenient surface.

In my experience, for dental undergraduates who I asked to do this simple test, the majority find no difference between using the sharp tip of the explorer and the blunt tip of the periodontal probe. The length of working end tip of most sharp caries explorers is less than 10mm, which makes it more difficult to access the root surfaces of deeper pockets.

The use of sharp probes has been shown to cause irreversible damage on enamel surfaces(Ekstrand *et al* 1987 ; Kühnisch *et al* 2007). Even in the hands of experienced clinicians, there is risk that the sharp tip of the caries explorer

can cause damage to the cementum when used to check for calculus on cementum as well as to the periodontium when inserted into the base of the periodontal pocket.

This author's preference is the periodontal probe, in particular the UNC-15. The periodontal probe is designed to be used safely in the periodontal pocket. The UNC-15 has a long enough working length to access even into deep pockets of up to 15mm.

In addition, the Nabers Probe is a useful probe to feel for calculus in furcation areas.

What does calculus feel like with the probe?

The first step to being able to detect dental calculus with a probe is knowing how it feels like.

In the past, where clinicians carried out root planing instead of root debridement, it may be sufficient to assume that any surface that does not have a "glass like" smoothness and hardness to it is calculus/diseased cementum complex and requires further planing until one feels the smooth hardness of dentine.

This is no longer the case.

What calculus feels like with the probe depends on the morphology of calculus present.

Calculus can have a surface topography like a craggy mountain range (figure 1). The surface could be burnished during root debridement and even feels smooth on the tactile examination, like a hump or bump (figures 2 & 4). If a root surface is covered with a layer of calculus, it could feel uneven (figure 3). If small specks of calculus are left on the root surface, it could feel like small protrusion on a flat surface (figure 4). A very mineralised sheet of calculus could have a surface that feels like sandpaper (figure 4). Cementum feels like smooth plastic.

Angle and movement of the periodontal probe on the root surface

The periodontal probe should be angled slightly against the root surface (figure 5) compared with how it is normally angled while using it to check probing pocket depths. This slight angulation allows the clinician to glide the tip of the probe along the root surface for tactile feel.

The movement should be either downwards, upwards and a slow up and down. The probe tip should always be in

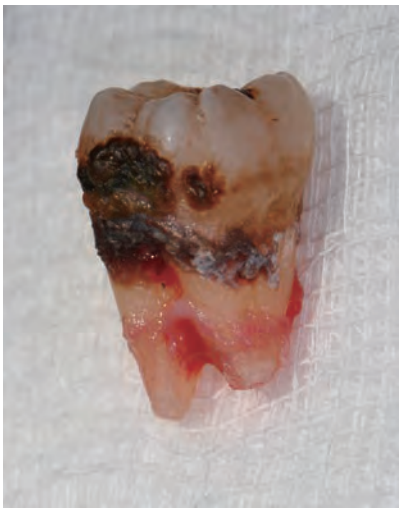


Figure 1 Calculus can have a surface topography like a craggy mountain range.

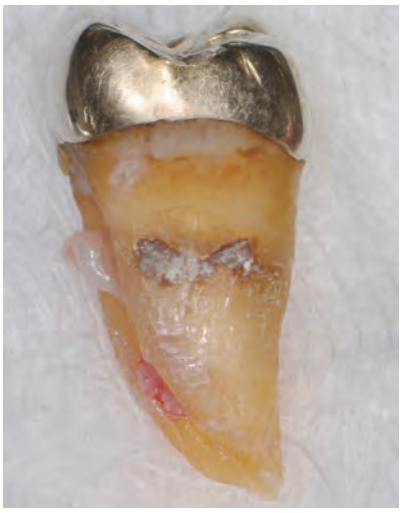


Figure 2 Calculus can feel like a hump or bump.

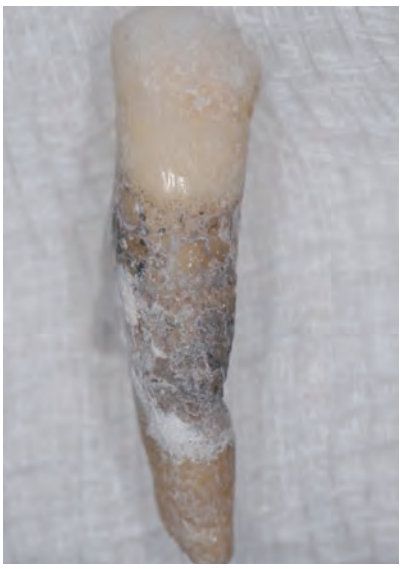


Figure 3 If a root surface is covered with a layer of calculus, it could feel uneven.

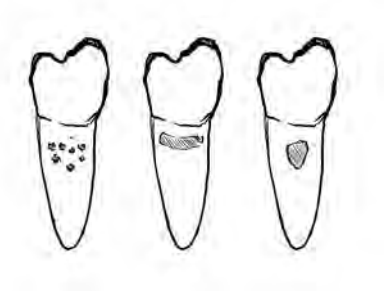


Figure 4 Calculus feel like small protrusions on a flat surface, a hump or like a sheet of sandpaper.

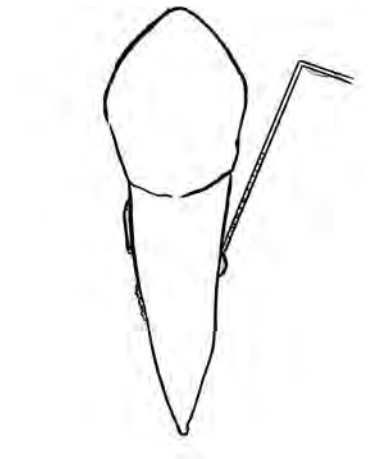


Figure 5 The periodontal probe should be angled slightly against the root surface such that the tip of the probe is in light contact with the tooth.

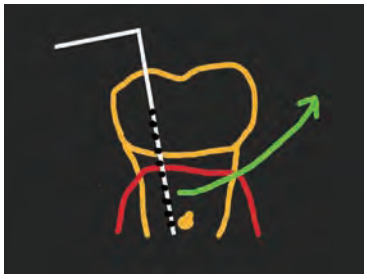


Figure 6 The probe can also be moved in a diagonal sweeping manner beneath the contact area to cover all the root surfaces.

contact with the root surface to pick up any catch, bumps, small protrusions or surface roughness.

Dental calculus often forms parallel to the cemento-enamel junction. The movement of the probe should therefore not be a lateral one as the tip of the probe could run on the crest of a calculus hump and it will feel smooth and regular. It is only by running the probe downwards and upwards along the long axis of the tooth that the bump/hump of the calculus can be picked up by the probe.

When the probe is run downwards along the root surface into the pocket and hits an obstruction that feels hard, many inexperienced clinicians may stop probing further downwards and think that the probe has reached the base of the probing pocket. The base of the probing pocket is made of connective tissue and offers a soft resistance, not a hard obstruction. This hard obstruction the probe feels is dental calculus, and the clinician should run the probe over the calculus, which often feels like a hump or craggy mountain top, until the probe navigates beyond the calculus deposit.

A feather-light touch pressure should be applied when running the probe along the root surface. The probe should be controlled with finger movements rather than using the wrist, which would give better control and tactile feel.

Buccal, lingual and line angles

For the buccal and lingual surfaces of the roots, the periodontal probe should be used as described above in a downward and upward movement along the long axis of the tooth. The probe should also be placed at the line angles of the tooth and moved in an up and down direction along that line.

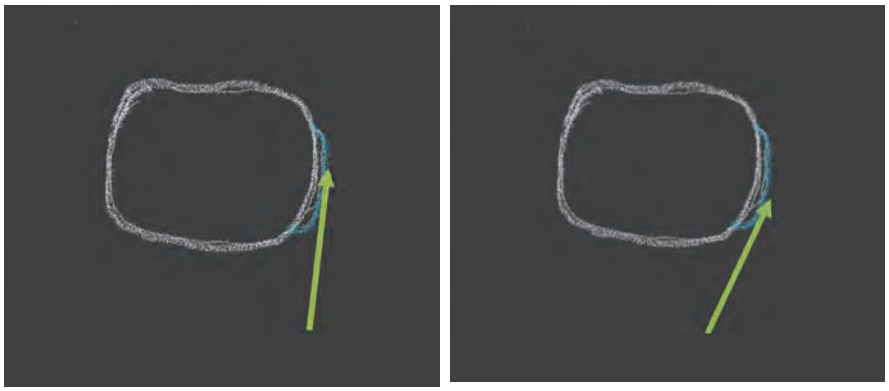
Interdental areas

At the interdental areas, the probe can also be used in an up and down direction as above. However, because of the contact area between two adjacent teeth, the probe has to be moved in a diagonal direction in order to examine the area of the root beneath the contact point (figure 6). In addition to an up and down movement, the probe can also be moved in a sweeping manner to try to examine all the root surfaces (figure 6).

The probe can also be positioned perpendicular to the long axis of the tooth to be inserted into the interdental space, and the side of the first 2mm of the working tip can be used to detect calculus. Because the tooth is not flat but a curve, it is important that the probe is continually adjusted as it moves from the line angle towards the contact area so that the probe is constantly positioned tangential to the curvature of the tooth, so that the sides of the probe tip is always in contact with the tooth (figures 7 & 8).

Furcations

The Nabers Probe can be used to access and feel root surfaces within the furcation which the periodontal probe is unable to reach.



Figures 7 & 8 Because the tooth is not flat but a curve, it is important that the probe is continually adjusted as it moves from the line angle towards the contact area so that the probe is constantly positioned tangential to the curvature of the tooth, and the sides of the probe tip is always in contact with the tooth.

Using visual with aid of magnification

With the aid of a high enough magnification (4x magnification) this author has also found that it is often possible to see sub-gingival calculus by pushing the gingiva slightly away from the tooth with the scaler tip while using an ultrasonic instrument. The water irrigation from the scaler helps flush away blood and keeps the field clean to assess the root surface visually.

Conclusion

Checking for sub-gingival calculus with the periodontal probe is like a golf swing. In the golf swing, the minor details matter. Likewise, the minor details of how to move the probe and keep the tip of the probe in contact with the root surface matter.

They both seems simple and intuitive but are not. Both the golfer and the clinician will benefit from clear instructions. The golfer can eventually 'feel' the strike of the ball with hours of practice, and the competent clinician the awareness of the presence of sub-gingival calculus.

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Effect of Mongolian Mumie Extract On Periodontal Condition

Oyunkhishig Khishigdorj, Bolortsesteg Dugersuren, Bayarchimeg Batbayar

Department of Periodontics & Endodontics, School of Dentistry, MNUMS "Iveelt dent" Dental Clinic

Keywords: Mumie, Baragshun EN, pocket dept, Gluco Chex

Abstract: Mumie is widely used in traditional Mongolian medicine from ancient times. This pale brown to dark brown tar-like substance is termed there Baragshun. Periodontitis is one of the highest prevalent dental diseases among Mongolians. The objective of this study was to evaluate the clinical effect of the Mumie Baragshun-EN for patients with chronic periodontitis.

In 20 patients, a clinical trial was conducted to compare effectiveness in bleeding on probing, pocket depths and gingival inflammation treated with Mumie EN. Probing depth (PD), bleeding on probing (BOP) clinical attachment level (CAL), and gingival inflammation were recorded before the treatment and 1, 3 months after treatment.

Before the treatment, the measurements of clinical parameters were similar for both groups. After scaling and root planing on periodontitis patients, greater reduction of pocket depths was shown in group with Mumie Baragshun-EN extract. Gain in CAL was also significantly greater in group with Mumie Baragshun-EN extract. BOP also reduced significantly.

The result of this study showed that the scaling and root planing with Mumie Baragshun- EN extract irrigation treatment was good and effective.

Introduction

Mumie has been widely used in traditional Mongolian medicine since ancient times. This pale brown to dark brown tar-like substance is termed as the "*Baragshun*", which means mountain juice. It is found as deposits in caves and rocky crevasses of high mountains in Mongolia and other parts of Central Asia. This traditional drug is also known as Mumie or Mumijo. In Ayurvedic medicine, Mumie is termed as Shilajit(Suraj 2007). Mumie contains fatty acids, benzoic acid, vitamins, such as B1 and B12, and other antioxidant compounds(Wilson *et al* 2011). Baragshun-EN was also found to have bacteriostatic and antibiotic-likeness activities and potent anti-inflammatory activity in all three models of acute, subacute and chronic inflammation(Enkh-Oyun and Narangerel 2014). Mumie is claimed to possess striking healing effects and protective influence on the human body and accelerates the healing of bone fractures(Wilson *et al* 2011). Periodontitis is an infectious disease that causes destruction of the tissues supporting the teeth. In Mongolian National Survey conducted by the Mongolian Association of Periodontology in 2021 showed the percentage of people with tooth loss was increasing by age from 29% in 15 years old to 82% in 65-75 years old groups. It was showed that people suffering from chronic periodontal disease since younger ages. The main objective of periodontal therapy is to improve the periodontal health and to preserve the dentition. In clinical practice, most forms of moderate periodontal disease are treated by scaling and root planing (SRP). It has been found that in adults with chronic periodontitis, scaling and root planing along with the use of an adjunctive antiseptic, antibacterial agents as a subgingival irrigation improves patient outcomes over a period of time compared to scaling alone(Bonito *et al* 2005). Subgingival irrigation can be performed by different agents such as water, saline, and antiseptics/antimicrobial agents(Chaudhari *et al* 2019). Various herbal extracts are used for local drug delivery as a comparatively safer alternative to synthetic compounds. Subgingival irrigation using herbal extract such as Mentha piperita (Menthol), cemmiphora myrrha, carvuum carinni, and turmeric, curcumin have demonstrated significant improvement in gingival health(Tyagi *et al* 2021). As an antiseptic, chlorhexidine has been used effectively in the treatment of periodontal disease. The use of synthetic compounds, such as chlorhexidine digluconate and tetracycline, causes various problems such as staining of tooth surface, alteration of taste, and mucosal allergy(Tyagi *et al* 2021).The clinical use of herbal products for the treatment of periodontitis is common in developing countries such as a Mongolia.

Material and methods

A total of 20 patients with chronic periodontitis were selected for this study. They were divided into the test group mumie, Baragshun EN extract (Group I) and the control group Gluco Chex 2% Gel (Group II), with 10 subjects in each group.

Inclusion Criteria: Subjects with chronic periodontitis.

All 20 subjects were age-matched (30±42 years old). All patients diagnosed with chronic periodontitis with periodontal pockets of depth ≥5mm were selected.

Exclusion Criteria: Subjects with systemic diseases such as diabetes mellitus, hypertension, had used antibiotics or any other periodontal affecting drugs in the previous month, pregnant or lactating women, smokers, or individuals who were allergy to chlorhexidine gluconate and mumie, were excluded.

Clinical procedures

At the baseline visit, the clinical parameters such as bleeding on probing score (BOP), the pocket depth (PD) and clinical attachment level (CAL) were recorded by using a Williams periodontal probe.

In test group: Following local anesthesia, subgingival calculus was removed and the roots were thoroughly scaled and planed by hand and ultrasonic instruments. During the scaling and root planing procedure, treated area was irrigated by endodontic double side-vented syringe with 5 ml of 2% Baragshun EN extract local drug delivery (formulated by IVM-Laboratory of Pharmacy, Ulaanbaatar, Mongolia) used in the study. The syringe was gently inserted into the depth of periodontal pockets to assure delivery of irrigants. Repeated irrigation ensured that the irrigating solution filled up pockets for a period of 5 minutes. Patients were instructed not to change their oral hygiene habits or to take any other medication throughout the study period, and oral hygiene instructions were reinforced. Participants were recalled after 1 and 3 months, and clinical parameters were reassessed.

In control group: All the procedure was the same except the irrigant used was a commercially available Gluco Chex 2% Gel. (Manufacturer: CERKAMED Medical Company, Poland)

Statistical analysis All data were recorded in a standardized format and analyzed to assess the efficacy of Baragshun EN extract (Group I) and Gluco Chex 2% Gel (Group II) at 1 month and 3 months using the SPSS 25 version. Mean of standard deviation (SD) of parameters such as PD, BOP, and CAL parameters was computed and then the comparison between the study groups done using unpaired t-test. Within-group comparison was made using paired ttest. Test of significance was set at $P < 0.05$.

Results

The pretreatment probing pocket depths of test and control groups were 4.72 ± 0.46 mm (mean ± standard deviation) and 4.74±0.61mm respectively. Three-month post-treatment probing pocket depths of test and control groups were 3.83mm±0.41mm (mean ± standard deviation) and 4.08±0.60 mm respectively. The difference between pretreatment and post-treatment probing pocket depths was found to be statistically significant in both test group (paired t-test value is 10.3 at 0.000 probability) and control group (paired t-test value is 6.73 at 0.000 probability).

Pretreatment CAL in test and control groups were 1.53±0.30 and 1.56±0.22 mm respectively. Post-treatment CAL in test and control groups were 1.03 ± 0.14 mm and 1.22 ± 0.16 mm respectively. The difference between pretreatment and post-treatment CAL was found to be statistically significant in test group (paired t-test value is 5.92 at 0.000 probability). The difference between pretreatment and post-treatment CAL was found to be statistically significant in control group (paired t-test value is 11.1 at 0.000 probability).

Pretreatment BOP scores of test and control groups were 0.90 ± 0.31 and 0.70 ± 0.48 respectively. Post-treatment BOP scores of test and control groups were 0.10±0.31 and 0.20±0.42 respectively. The difference between pretreatment and post-treatment BOP was found to be statistically highly significant in test group (paired t-test value is 6.00 at 0.000 probability). The difference between pretreatment and post-treatment BOP was found to be statistically significant in control group (paired t-test value is 3.00 at 0.015 probability). The results are depicted in Table.1 and Graphics 1 to 3.

The pretreatment probing pocket depths of test and control groups were 4.74±0.61mm (mean ± standard deviation) and 4.72±0.46 mm respectively. Difference between test and control group was found to be statistically nonsignificant at 0.34 probability (unpaired t-test score was 1). Three- month post-treatment probing pocket depths of test and control groups were 3.83±0.41mm (mean± standard deviation) and 4.08±0.61mm respectively. Difference between test and control group was found to be statistically nonsignificant at 0.59 probability (unpaired t-test score was -0.59).

Pretreatment clinical attachment loss in test and control groups were 1.53±0.29 and 1.56±0.22 mm respectively. Difference between test and control group was found to be statistically nonsignificant at 0.82 probability (unpaired t-test

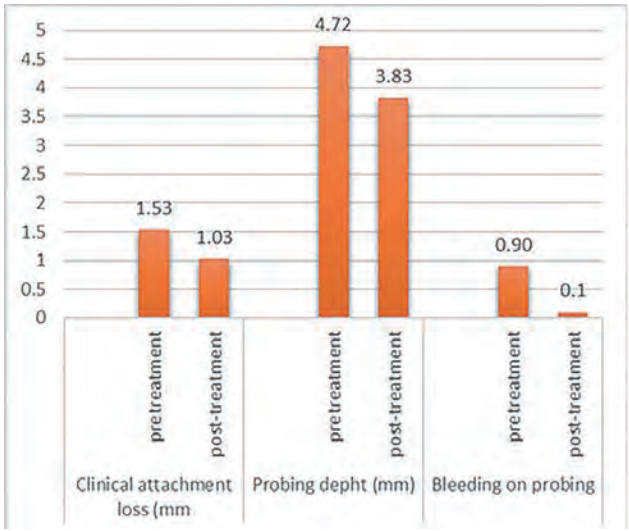
score was -0.23). Post-treatment clinical attachment loss in test and control groups were 1.03±0.46 mm and 1.22±0.16 mm respectively. Difference between test and control group was found to be statistically significant at 0.08 probability (unpaired t-test score was -1.92).

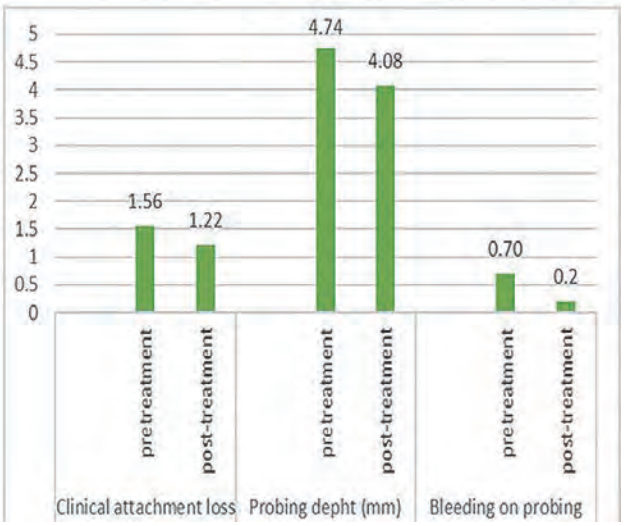
Pretreatment bleeding on probing scores of test and control groups were 0.90±0.3 and 0.70 ± 0.48 respectively. Difference between test and control groups was found to be statistically nonsignificant at 0.93 probability (unpaired t-test score was 0.93). Post-treatment bleeding on probing scores of test and control groups were 0.10±0.31 and 0.20±0.42 respectively. Difference between test and control groups was found to be statistically nonsignificant at 0.17 probability (unpaired t-test score was -1.47). The results are depicted in Table.2.

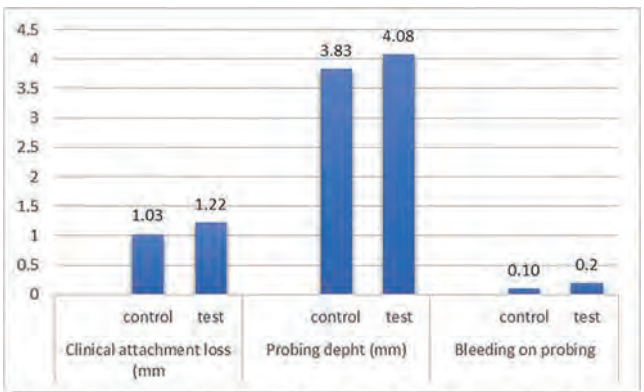
Discussion

Recently, the use of sustained release formulations to deliver antimicrobial agents to the site of infection in periodontal pockets has been reported successful for treating periodontitis(Pragati *et al* 2009).

The present clinical study does not permit comparisons with any other studies. There are no studies analyzing the adjunctive effect of mumie extract in periodontal treatment such as SRP in patients. The present study used mumie Baragshun EN as a subgingival irrigant. We have not observed reactions such as abscesses or allergic reactions. The BOP values also improved significantly ($p<0.001$) compared with baseline data. Results of this study show that the reduction in PD and in CAL were more when irrigated with Baragshun EN extract. Reduction in PD score was better in Baragshun EN extract group as compared with







Graph.3. Comparison of poste treatment mean values of different parameters between control and test groups

chlorhexidine group at all the follow-up periods for up to 90 days, which were similarly observed with studies by Shweta S. and Hugar.et al, comparing chlorhexidine gel and curcumin gel as an adjunct to scaling and root planing. In the present study, the effect of single irrigation was evaluated, which is in accordance with the studies by Taggart et al., Amritpal Kaur et al, and Christersson et al. observing significant improvement with single irrigation. The subgingival scaling and root planing were carried out before the irrigation and plaque score was brought to zero at baseline. However, the present results do not have sufficient data in this short-term follow-up, and microbiological evaluation was not done. To validate mumie Baragshun EN as a local drug delivery system, further long-term studies with a larger sample size are required to analyze its clinical and microbiological efficacy in comparison to other available local drug delivery products in the treatment of chronic periodontitis.

Conclusion

In this study, 10% Baragshun EN extract irrigation as an adjunct to SRP nonsurgical periodontal therapy has shown slightly better results due to its antiseptic and antimicrobial activity. It could be considered as an adjunctive treatment approach in the treatment of chronic periodontitis.

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Table1.Pre&post-treatment values of different parameters for test and control groups

Groups	Pretreatment Mean ±SD	Post-treatment Mean ±SD	t-value	P value	Significance
Control group					
Probing depth (mm)	4.74±0.61	4.08±0.61	6.73	0.000	HS
Clinical attachment loss(mm)	1.56±0.22	1.22±0.16	11.1	0.000	HS
Bleeding on probing	0.70 ± 0.48	0.20±0.42	3.00	0.015	S
Experimental group					
Probing depth (mm)	4.72±0.46	3.83±0.41	10.3	0.000	HS
Clinical attachment loss(mm)	1.53±0.29	1.03±0.46	5.92	0.000	HS
Bleeding on probing	0.90 ± 0.31	0.10±0.31	6.00	0.000	HS

NS:Nonsignificant; S:Significant; HS:Highly significant

Table2.Comparison of different parameters in test and control groups

Character	Control group	Test group	t-value	P value	Significance
Before the treatment					
Pocket depth (mm)	4.74±0.61	4.72±0.46	1	0.34	NS
Clinical attachment loss(mm)	1.56±0.22	1.53±0.29	-0.23	0.82	NS
Bleeding on probing	0.70 ± 0.48	0.90 ± 0.31	-0.08	0.93	NS
3 months after treatment					
Pocket depth (mm)	4.08±0.61	3.83±0.41	-0.55	0.59	NS
Clinical attachment loss(mm)	1.22±0.16	1.03±0.46	-1.92	0.08	S
Bleeding on probing	0.20±0.42	0.10±0.31	-1.47	0.17	NS

NS:Nonsignificant; S:Significant; HS:Highly significant

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Periodontitis and Diabetes: the Malaysian Multidisciplinary Approach

Rathna Devi Vaithilingam

Universiti Malaya, Kuala Lumpur, Malaysia

Introduction

Associations between periodontal disease and systemic diseases have been reported in the literature over the past three decades. Numerous diseases have been linked to periodontal disease such as Type 2 diabetes mellitus (T2DM), respiratory disease, cardiovascular disease, preterm low birth weight pregnancy, rheumatoid arthritis and many others. Most of these associations have been mainly based on observational studies(Monsarrat *et al* 2016). In 2017, Bartold and Mariotti investigated the current evidence on various periodontal and systemic inter-relationships using a "disease association check list" based on common risk markers and experimental data that define the disease (Bartold and Mariotti 2017). Amongst these diseases, T2DM was the only disease that was found to show biologic plausibility, strength of association, effect of periodontal therapy on disease condition and effect of treatment of disease condition on periodontal disease.

Diabetes prevalence and health-care cost

The worldwide prevalence of adults diagnosed with diabetes is increasing rapidly. In 2021, 537 million people (20-79 years) globally were living with diabetes with total expenses incurred being USD966 billion. By 2045, the projected numbers are expected to rise to 783 million (https://www.diabetesatlas.org). In Malaysia, more than 4.4 million people have been diagnosed with diabetes in 2021 with the projected number in 2045 being about 6.5 million adults. Total Malaysian health-care cost of diabetes in 2017 was RM 4.38 billion whereby 70.56% of the cost was attributed to primary care and outpatient attendances (70.56%)(Ministry of Health Malaysia 2022).

Diabetes as a risk for periodontitis

Data from epidemiologic studies have confirmed that T2DM plays a major role as a risk factor for periodontitis, as susceptibility to periodontitis is increased in people with T2DM (Preshaw *et al* 2012). The incidence rate of periodontal disease in subjects with T2DM is 2.6 times (95% Cl 1.0-6.6, controlled for age and sex) compared with those without the disease (Nelson *et al* 1990 ; Taylor *et al* 1998). Severity of T2DM is greater in all ages and increases with age. Severity also increases in individuals with uncontrolled T2DM. The disease was also associated with increased rate of alveolar bone loss progression over a period of 2 years.

Periodontitis as a risk factor for T2DM

Severe periodontitis has been shown to cause a 0.29% increase in HbA1c levels (95% CI 0.20, 0.27) and increased odds of developing incident T2DM (adj HR 1.19-1.33)(Graziani *et al* 2018). T2DM patients with severe periodontitis have also been found to have higher risk of cardiovascular mortality and morbidity, and increased mortality in patients with diabetic kidney disease(Sanz *et al* 2018 ; Sharma *et al* 2016 ; Southerland *et al* 2012). The link between periodontitis and T2DM has been postulated to the increased systemic load of the circulating inflammatory mediators caused by periodontitis which causes impairment of insulin signaling and insulin resistance, thereby causing an exacerbation of T2DM (Preshaw *et al* 2012).

Effect of periodontal therapy

Periodontal therapy reduces the systemic inflammatory load caused by periodontitis and therefore reduces insulin impairment, and a beneficial improvement is seen in diabetic control (Preshaw *et a*l 2012 ; Raman *et al* 2014). The latest Cochrane database systematic review on the effect of non-surgical periodontal therapy on diabetic control has found a reduction in HbA1c levels of 0.43% at 3 to 4 months and 0.5% at 12 months post-treatment (Simpson *et al* 2022). This reduction was even seen in poorly controlled diabetics, and the adjunctive use of antibiotics did not enhance reduction beyond scaling and root surface debridement (Sanz *et al* 2018 ; Madianos and Koromantzos 2018).

As ample evidence supports the link between T2DM

and periodontitis, closer collaboration between physicians and oral health care professionals is warranted to improve glycaemic control (Sanz *et al* 2018). Additionally, there is a need for healthcare providers to routinely refer these patients for oral healthcare as part of the holistic care for people with T2DM (Sahril *et al* 2014 ; Salleh *et al* 2017). A recent study conducted in 2018 looking into the awareness, knowledge, attitudes, and practices on the management of diabetes patients with periodontitis amongst Malaysian primary care practitioners reported that although medical practitioners had good knowledge about periodontal disease, a vast majority (83.1%) claimed that it was not their responsibility to ask or examine diabetic patients for symptoms of periodontal disease (Nordin *et al* 2021). When primary care practitioners were asked about the barriers faced in referring their patients to their dental colleagues, reasons given were patient's refusal for the referral, heavy workload, time constraints in writing referral letters and oral healthcare not being part of diabetes management as stated in the Clinical Practice Guidelines (CPG) produced by the Ministry of Health Malaysia and followed closely by medical practitioners who were managing patients with T2DM. Nordin et al. concluded that this information should be incorporated in the Malaysian CPG on management of T2DM patients(Nordin *et al* 2021).

Clinical Practice Guidelines on T2DM management

In 2019, a taskforce for the 6th edition of the CPG on T2DM management was set-up to review and update the 5th edition (Ministry of Health Malaysia, 2020). The chairperson of the task-force had emphasised that T2DM is not a stand-alone disease but instead was reaching across a spectrum of other noncommunicable diseases. Hence there was a need to include other specialities in the updated guidelines. Thus, for the first time, periodontal disease was included in the guidelines as a complication of T2DM.

The recommendations for 'Periodontal disease in T2DM' given in the CPG are:

Oral health education should be provided to all patients with T2DM emphasizing the increased risk of periodontal disease in T2DM. Successful management of periodontal disease may improve metabolic parameters.

Physicians/medical health professionals should investigate the presence of periodontal disease as an integral part of T2DM care visits. If present, prompt referral should be made to the dentist for periodontal examination.

For all people with newly diagnosed diabetes, referral for a periodontal examination should occur as part of their management. Even if no periodontitis is diagnosed initially, annual periodontal review is recommended.

With the implementation of the updated 6th edition of the Malaysian CPG on the management of T2DM, it is hoped that the medical and health care professionals work together to provide whole-person care for patients with periodontal disease and T2DM. The improvements seen following periodontal therapy will help reduce the national economic burden posed by T2DM.

Conclusion

The inflammatory link between periodontitis and T2DM emphasises the important role that physicians and dentists play in managing both conditions. With the inclusion of periodontal disease as a complication of T2DM in the current CPG for T2DM in Malaysia, the awareness has been created for improved collaboration and referrals between the medical and oral healthcare professionals, to assist patients trying to traverse the medical-dental divide and to provide optimal patient-centered care.

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Periodontal Therapy, Aging Society, and COVID-19 Pandemic: Bridging Basic Research to Clinical Setting

Benso Sulijaya [1,2*], Fatimah Maria Tadjoedin[1], Sandra Olivia Kuswandani[1,3], Yuniarti Soeroso[1]

[1] Department of Periodontology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia.
[2] Universitas Indonesia Hospital, Universitas Indonesia, Depok West Java. Indonesia.
[3] Graduate student program, Periodontology Unit, Eastman Dental Institute. London, England, United Kingdom.
[*] Corresponding Author: bensosulijaya@gmail.com; benso.sulijaya87@ui.ac.id

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Abstract:

Background: A recent approach to treating periodontitis involves resolving inflammation and oral microbiome dysbiotic conditions. Periodontitis is reported to affect 74.1% of the population in Indonesia. The prevalence of periodontitis is higher in the aging community, with 64% of the elderly (>65 years) having moderate to severe periodontitis. The continuous spread of the coronavirus disease-19 (COVID-19) and Omicron variants has affected host inflammatory response, resulting in fatal outcomes in vulnerable subjects, such as the elderly. Thus, the periodontal inflammation associated with COVID-19 in the aging society becomes a concern. The present review aimed to elaborate on the inflammatory response and oral pathogens in the aging society, confirm underlying mechanisms, and propose a novel approach to manage periodontitis during the COVID-19 pandemic. Accumulating data suggest that the oral hygiene index (OHI) was higher in the elderly than in adults, although the periodontal probing depth (PPD) was similar. Although the TNF-a levels were not significantly different, the elderly group showed an abundance of red complex pathogens (*P. gingivalis* 2.5-fold, *Tannerella forsythia* 3-fold, *Treponema denticola* 10-fold) compared to adults ($p>0.05$). Furthermore, the proliferation rate in gingival tissue cells of adults and old rodents showed no significant difference between the two groups. Both cells produced epithelial barrier function-related genes equally towards the *P. gingivalis* challenge. In addition to COVID-19, Perio-UI mobile apps increased oral health awareness (cognitive and behavior), improved OHI, and reduced bleeding on probing (BOP) in patients with periodontitis. Overall, periodontitis and COVID-19 share inflammatory reaction characteristics. No significant alteration in the oral microbiome and gingival epithelial barrier function was present in the elderly; however, inflammatory biomarkers could have elevated. Further, periodontitis could be a risk factor for COVID-19 as patients with severe periodontitis showed worse COVID-19 complications. A mobile-based app is preferred for teledentistry to increase oral health awareness and maintain oral symbiotic conditions.

Periodontitis: The Present

Increasing data suggest that oral bacterial dysbiosis, which causes chronic low-grade inflammation of the dental supporting tissue, is the cause of periodontitis (Nwizu *et al.* 2020) and consequent alveolar bone loss (Di Benedetto *et al.* 2013; Yu and Van Dyke 2020). Interestingly, the concept is that periodontitis development is not caused by specific bacteria in dental plaque, implying that dental plaque is essential but insufficient to cause periodontal disease (Yu and Van Dyke 2020). Bacterial challenge initiates inflammation; however, uncontrolled production of pro-inflammatory cytokines and mediators may be responsible for periodontal disease pathogenesis and tissue breakdown (Yu and Van Dyke 2020; Bartold and Van Dyke 2019). Clinical studies have shown that the bacterial load does not necessarily correlate with the intensity of this damage, but there is no dispute about the role of bacteria in the initial pathogenesis of periodontitis, including the presumed immunostimulatory components (Cekici *et al.* 2014). Moreover, inflammation may result from bacterial dysbiosis and oxidative stress conditions (Hajishengallis and Lamont 2012; D'aiuto *et al.* 2010).

Global prevalence rates for periodontal disease, particularly its mild and moderate forms, are over 50% in adult populations (Eke *et al.* 2012), while rates for the severe form, which tends to increase between the 30th and 40th year of life, are roughly 10% (Kassebaum *et al.* 2014). In 2017, 796 million people were affected by periodontitis, with a 9.8% prevalence of severe periodontitis (Collaborators *et al.* 2020). Reports show that periodontitis prevalence is 74.1% in the Indonesian population, based on the 2018 Indonesia National Health Survey (RISKESDAS) (Santoso *et al.* 2022). Age, sex, race, and socioeconomic factors are among the demographic variables that affect the incidence of periodontitis. Smoking, diabetes mellitus, metabolic syndrome, and obesity are other significant risk factors (Genco and Borgnakke 2013).

Chronic and aggressive are no longer used terms for periodontitis based on the latest classification (Tonetti *et al.* 2018) due to the lack of specific pathophysiology that allows for the distinction between aggressive and chronic periodontitis and no clear direction for various therapies. Moreover, the average progression rates of periodontitis are constant worldwide among all populations observed. Currently, periodontitis is classified into staging (based on severity and complexity) and grading (according to disease progression, impact on systemic health, and response to the treatment) (Tonetti *et al*. 2018). Regarding the therapy, Sanz et al. (2020) reported the S3-level clinical practice guidelines by the European Federation of Periodontology (EFP) for managing periodontitis stage I-III (Sanz *et al.* 2020). The first phase of therapy is to direct behavior modification by inspiring the patient to complete successful supragingival dental biofilm clearance and risk factor reduction. Eliminating the subgingival biofilm and calculus is the goal of the second stage of treatment (cause-related therapy) (subgingival instrumentation). The third step is to gain better access for subgingival instrumentation, defect regeneration, or resection that did not respond to step two of therapy. Lastly, all patients with treated periodontitis receive supportive periodontal treatment to maintain periodontal stability (Sanz *et al.* 2020).

Periodontitis in Elderly

Periodontitis has become more prevalent in the aging population, with 64% of the elderly (>65 years) having moderate to severe periodontitis (Tadjoedin *et al.* 2017). The number of people worldwide who are 60 years or older is increasing with increased life expectancy. By 2050, the number of senior citizens is estimated to be 2.1 billion (Kanasi *et al*. 2016). With 15.8% of the population expected to be 60 and older by 2035, Indonesia will likewise experience an aging population that will influence the rise of age-related diseases and conditions (Patterson 2018). Interestingly, a study showed that only 3% of the 369 older adults in Indonesia had healthy periodontal status, implying that the remaining subjects suffered from periodontitis (Hijryana *et al* 2022). Moreover, age and periodontal disease often go together. A study showed an association between age-at-onset and pattern of disease progression (Tadjoedin *et al.* 2017).

Subgingival biofilms act as reservoirs of anaerobic gram-negative bacteria (Socransky and Haffajee 2005). Likewise, several microorganisms, such as *Fusobacterium, Campylobacter, Prevotella, Capnocytophaga, Selenomonas*, and *Actinomyces,* have been investigated for their associations with periodontitis [19]. *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) may produce enzymes that digest extracellular matrix components and facilitate tissue invasion (Page and Schroeder 1976). Moreover, human studies have reported virulence factors of these bacteria (Chaves *et al.* 2000). In terms of complexes, the orange complex bacteria can adhere to other bacteria and connect commensal populations; they are typically periodontal pathogens, referred to as "connecting" organisms, and are crucial for the survival of the red-complex bacteria[6]. A study by Tadjoedin et al. (2022) involving elderly subjects showed an association between periodontitis and cognitive impairment through orange and red complex bacteria alteration (Tadjoedin *et al.* 2022).

A recent study by Sumbayak et al. (under review-2022) found that oral hygiene index score, plaque index, and papillary bleeding index (PBI) in the elderly were significantly higher than in adults. The pro-inflammatory cytokine IL-1b was significantly upregulated in elderly subjects. Meanwhile, TNF-a levels and IL-10 tended to increase in the elderly but were insignificant. All findings showed an inflammatory response due to cognitive and psychomotor limitations in the elderly and possible subgingival microbial alteration. Khoirowati et al. (under review-2022) performed a bacterial analysis investigation to prove this hypothesis by comparing the red complex bacteria in the elderly and adults. Interestingly, compared to adults, elderly subjects expressed a 2.5-fold higher level of *P. gingivalis*, 10-fold greater *T. denticola,* and three-fold greater *T. forsythia.* Taken together, aging might alter the subgingival environment, thus affecting the immune response.

Epithelial Barrier Function Regulates Periodontitis Progression

The gingival epithelium provides mechanical and chemical barriers between the oral environment and the underlying tissue against foreign pathogens and their products (Amano 2007; Takahashi et al. 2019). Periodontal disorders start and progress due to epithelial cell-to-cell contact disturbance, termed "leaky gum"(Park *et al.* 2022).

In more detail, *P. gingivalis* may break down the tight junction protein (*occludin*) and adhesion junction proteins (*E-cadherin*), boosting bacterial paracellular translocation (Katz *et al.* 2000) with an increased attachment of fimbriae to oral epithelial cells[23]. Regarding the microorganisms, cells from the epithelium release pro-inflammatory cytokines and chemokines, which cause immune cells to secrete local inflammatory mediators, such as IFN- and TNF- (Miyamoto *et al.* 2015). Studies have shown that these host immune mediators affect the gingival epithelial barrier function.

An in vitro investigation demonstrates that treatment with bacterial lipopolysaccharide (LPS) decreases the production of *claudin-1* in the junctional epithelium, preventing the rupture of the epithelial barrier (Fujita *et al.* 2012). Other research has shown that the outer membrane protein of *A. actinomycetemcomitans* lowers *connexin-43* levels (Fujita *et al.* 2006; Fujita *et al.* 2008; Uchida *et al.* 2005). In the gingival epithelial cells, *P. gingivalis* and *A. actinomycetemcomitans* suppress the production of *E-cadherin* (Katz *et al.* 2000; Noguchi *et al.* 2003). Recently, a report showed that a *P. gingivalis* virulence factor reduced E-cadherin protein in gingival epithelial cells, disrupting the activities of the epithelial barrier *in vitro*. An in vivo study proposed that the etiology of periodontitis involved E-cadherin degradation (Yamada *et al.* 2018).

A recent study by Mutiara et al. (under the proofread-2022) shows that aging affects epithelial barrier function. In detail, cells collected from two different age groups (18 and 58-week-old mice) showed similar proliferation and cell metabolism activity. Hence, cell proliferation in the young showed gradual growth, while the older subjects showed a fluctuating growth pattern. Further, the expression of epithelial barrier function-related genes (*ZO-1, occludin, JAM-A,* and *claudin*) toward the *P. gingivalis* challenge was significantly downregulated in the older subjects compared to the young. This finding suggests that the elderly are more vulnerable to periodontitis.

The direct effects are primarily mediated by the microorganisms or their products altering barrier function-related genes/proteins, while the indirect effects are predominantly regulated by the interaction of host cellular immune response to bacterial challenge (Turner 2009). Thus, epithelial integrity regulates the initiation and progression of periodontitis.

Periodontitis and COVID-19

Meanwhile, the world continues to suffer from COVID-19, rising again due to the Omicron variant (Meo *et al.* 2021; Covid and Team 2021; Pitones-Rubio *et al.* 2020). COVID-19, a viral infection that clinically affects multiple human organs, is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Cells in the oral cavity produce angiotensin-converting enzyme 2 (ACE-2), which is related to tissue inflammation and aging and promotes viral replication. An immunohistochemical study by Sakaguchi et al. (2021) showed differences in ACE-2 expression between the squamous epithelium layers of the tongue. Real-time polymerase chain reaction (RT-PCR) has revealed ACE-2 in the epithelial cells of taste buds. Moreover, the squamous gingival epithelium expressed ACE-2, primarily in the cytoplasm and nucleus of the spinous basal layer. The ductal epithelium and the serous cells of the submandibular gland express ACE-2 (Sakaguchi *et al.* 2020). In addition to ACE-2, TMPRSS2 also binds to SARS-CoV2 (Senapati *et al.* 2021; Song *et al.* 2020). The periodontal pocket epithelium and the sulcular epithelium express ACE-2 and transmembrane protease serine 2 (TMPRSS2) (Sakaguchi *et al.* 2020). Targeting the receptor binding domain by administering ACE-2 blockers, soluble ACE-2, and TMPRSS2 inhibitors is the primary therapeutic strategy of SARS-CoV2-ACE2 binding methods. In conclusion, the SARS-CoV2-ACE2 binding interface is possibly the finest method for combating the virus (Sharifkashani *et al.* 2020).

COVID-19 infection affects host inflammatory response, resulting in fatal outcomes in vulnerable subjects, such as the elderly. In Indonesia, older people accounted for 38.6% of COVID-19-related deaths (Azwar *et al.* 2020), possibly due to bacterial co-infection because patients with severe COVID-19 have immune dysregulation, a higher abundance of *Prevotella intermedia, Streptococci, Fusobacterium,* and other infectious bacteria associated with acute periodontal disease development (Xiang *et al.* 2021; Botros *et al.* 2020; Marouf *et al.* 2021). The presence of periodontal pathogens, such as *Prevotella, Fusobacterium,* and *Treponema,* in broncho-alveolar fluid in COVID-19 subjects (Takahashi *et al.* 2021; Imai and Tanaka 2021) suggests the possibility of bacterial aspiration. Takahashi et al. (2021) showed that SARS-CoV-2 infection, through binding to the ACE-2 receptor, induces an intense, heightened inflammatory

response, known as cytokine storm, where pro-inflammatory cytokines IL-6, IL-8, IL-17, and TNF-alpha associated with periodontal disease contribute to the severity of COVID-19[46]. Marouf et al. (2021) demonstrated that patients with periodontitis had an odds ratio of 6.34 (95% CI, 2.79-14.61) for COVID-19 complications, 17.5 (95% CI, 2.27-134.8) for mortality, and 7.31 (95% CI, 2.21-26.3) for ventilation requirement[45]. Bacthiar et al. (2022) found ACE2 expression in the saliva of COVID-19 patients and suggested that its correlation with *C. albicans* and gram-negative oral bacteria may contribute to developing a COVID-19 severity predictor based on oral dysbiosis (Bachtiar *et al.* 2022).

Thus, the periodontal pocket provides a perfect niche or reservoir for SARS-CoV-2. The findings suggest that periodontal disease plays a role in the recurrence of COVID-19 because it enables the virus to replicate in the periodontal tissue. Virus from the saliva enters the bloodstream through the ulcerated periodontal tissue in the pockets and reaches distant organs (Botros *et al.* 2020; Badran *et al.* 2020). Various studies have shown several clinical oral symptoms characteristic in patients with COVID-19[45 (p19)].

Periodontal Treatment during COVID-19 Pandemic

The best periodontal therapy is mechanical debridement, which removes calculus and biofilm and fosters a healthy oral environment (Mombelli 2018). However, patients have limited access to treatment due to limitations in dental visits and regulation of aerosol-generating procedures during the COVID-19 pandemic. The American Dental Association released a statement on March 16, 2020, advising that elective treatments should be postponed for three weeks, with precedence to dental emergencies to avoid COVID-19 spread (Kranz *et al.* 2021).

SARS-CoV-2 spreads by direct routes, such as human-produced droplets and aerosols, or non-direct routes, including contact with infected objects and airborne contamination, and even with the operator's personal protective equipment (PPE). Droplets can often only reach a distance of two meters and remain contagious for a short time (Yuan *et al.* 2020). However, SARS-CoV2 can survive and spread by airborne droplets for up to three hours (Van Doremalen *et al.* 2020). SARS-CoV-2 is more contagious than its predecessors due to its distinct feature.

Figure1. PerioUICare Mobile Apps

These characteristics may contribute to the further spread of COVID-19 through dental treatment.

According to a poll in the United States, patient attitudes toward dental and oral health and dental treatment routine have changed due to concern and dread about the possibility of COVID-19 transmission. According to guidelines by the Indonesian Dental Association, there are restrictions on providing direct care for dental and oral health conditions during the COVID-19 pandemic (Widyarman *et al.* 2022). Restrictions on dental care combined with the dentist and patient concerns over COVID-19 transmission will affect how patients feel about going to the dentist for scaling and how prevalent periodontal disease is in Indonesia during the COVID-19 pandemic (Gao *et al.* 2020; Widyarman *et al.* 2020).

A survey by Sania et al. (data unpublished) showed the perception of 473 students toward periodontal treatment (scaling) during the COVID-19 pandemic. Although the study found no significant differences in male and female subjects, the data implied that 65.8% of students hesitated to receive periodontal therapy due to the possibility of COVID-19 transmission during the dental visit. In line with the findings, a study (Moffat *et al.* 2021) noted elevated patient concerns

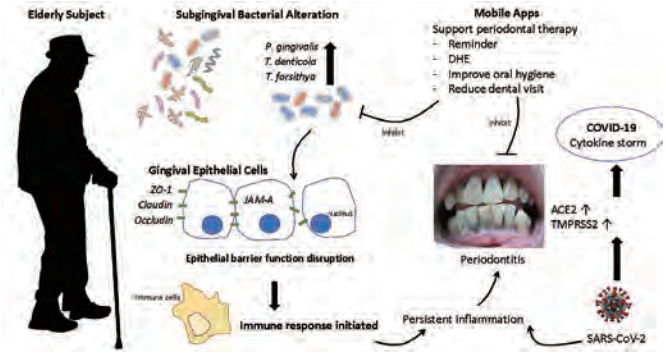

Figure 2. Illustration in regard to Aging, Periodontitis, and COVID-19

about COVID-19 (Moffat *et al.* 2021).

A recent study showed a novel protocol that a single dental selfie could effectively reveal periodontal status using the modified gingival index (Tobias and Spanier 2020). This study has resulted in a paradigm to support the need for new protocols or devices that can provide easy access to health for patients without having to visit in person, especially in distance-priority situations, such as COVID-19 (Telles-Araujo *et al.* 2020).

Teledentistry and Mobile Apps

In contrast to face-to-face interactions with patients, teledentistry uses information technology to facilitate remote dental care, counseling, education, or treatment. (Khan and Omar 2013) It is a subset of telehealth along with telemedicine. Teledentistry is widely used in dental practice in periodontology, malocclusion, endodontic problems, dental infections, dental caries, patient education, oral lesions, and diagnosis (Khan and Omar 2013). Implementing digital medical records, referral systems, image digitization, teleconsultations, and telediagnosis are a few modes and techniques in teledentistry (Ghai 2020). Further, teledentistry has gained popularity over the years for remote dental screenings, diagnosis, consultation, and treatment planning. In places with limited access to facilities, among schoolchildren, and in long-term healthcare facilities, teledentistry proves comparable to face-to-face consultations (Khan and Omar 2013; Estai *et al.* 2018). This concept seems feasible and legitimate for oral disease identification.

Healthcare delivery is becoming more effective thanks to the rising field of telemedicine/teletriage in the digitalization of healthcare. It overcomes geographical and socioeconomic barriers that previously prevented many people from access to treatment. In dentistry, teledentistry is a promising approach for assisting new, isolated, and rural healthcare professionals. A qualitative study and systematic review by Irving et al. (2018) examined a teledentistry project for precision, efficacy, or description in actual practice situations. Qualitative analysis revealed that teledentistry offers a practical and reasonable option for remote screening, diagnosis, consultation, treatment planning, and guidance. Advances in information and communication technology have improved access to accurate, efficient, and cost-effective remote help by physicians (Irving *et al.* 2018). Thus, teledentistry is well-accepted by both patients and clinicians.

The widespread use of mobile phones has become the standard in contemporary culture. A report showed that 91% of the population used cell phones in 2012. Moreover, more than 50% of the human population can easily connect to the Internet (Pearson *et al.* 2017). Mobile devices can promote and practice mobile health (mHealth) for public health interests (Rehalia and Prasad 2016; O'Leary *et al.* 2016). They have developed into a platform for encouraging patient-oriented self-care, improving patient-centered care, stimulating the growth of health literacy, and slanting the public perception of diagnosis favorably (Finkelstein *et al.* 2012; Tang *et al.* 2016; Diviani *et al.* 2015). According to studies, 31% of smartphone owners use their devices for health education, and 19% have applications downloaded to manage their health and well-being or learn more about a current medical problem (Fox and Duggan 2012). Another survey also revealed that more than 56% of healthcare provision settings employ mHealth to support clinical practice (Franko and Tirrell 2012).

A short message service (SMS) and digital reminders have been widely investigated for the mHealth approach. Research and empirical studies show significant evidence of mental health interventions utilizing mobile applications (Marks *et al.* 2003; Lippman 2013). Mental and physical health may improve in patient groups receiving SMS messages. SMS messages are convenient since they are sent directly to smartphones (Chen *et al.* 2008; Ybarra *et al.* 2013; Horvath *et al.* 2012).

A systematic review by Rathbone et al. (2017) investigated the role of mobile applications and SMS messaging

in physical and mental health (Rathbone and Prescott 2017). The review highlighted the importance of mHealth therapies in effectively addressing problems with physical and psychological health. The apps significantly impact medication adherence, and SMS-based psychoeducational material is easy to understand (Rathbone and Prescott 2017). Further, it shows that apps and SMS text messaging can reduce symptoms of stress, depression, and anxiety, encourage a healthier lifestyle, and promote adherence to medication. Thus, these approaches improve patient compliance and reduce visits to healthcare facilities and professionals (Clough and Casey 2015; Kelli *et al.* 2017). These mobile health interventions also enable patients to be responsible for their condition.

A randomized controlled trial by Hartono et al. (data unpublished) showed the clinical benefits of an android-based mobile app, PerioUICare, to support periodontal treatment (Fig. 1). This app accommodates patient compliance, periodontal status, diagnosis, treatment plans, and prognosis of the tooth. Further, it provides dental health educational content as a regular reminder adjusted to patient needs and status.

Conclusion

As summarized in Fig. 2, periodontitis is associated with COVID-19 infection because vulnerable patients, such as elderly subjects with severe periodontitis, may experience worse COVID-19 consequences. We believe a mobile app can supplement the compromised face-to-face dental treatment in pandemics, maintain oral bacterial symbiosis conditions, and raise oral health awareness through digital engagement using the teledentistry approach. All teledentistry applications work to increase efficiency, reach marginalized populations, boost service quality, and lessen the burden of oral diseases.

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Conflict of Interests

The authors declare no potential conflict of interests in this study.

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Abstracts of Poster Presentation Awards

Clinical research

First prize

Title: Evaluation of metallothionein and selected pro-inflammatory cytokines in saliva of periodontitis patients: a pilot study

Presenter: Jan Yang Ho

Authors: Jan Yang Ho[1], Nur Falihin Soehairq Kamal Nurzaman[2], Nor Shafina Mohamed Nazari[1], Mohammad Tariqur Rahman[3], Nor Adinar Baharuddin[1]

[1] Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia; [2] Primary Care Unit, Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia; [3] Faculty of Dentistry, University of Malaya, 50603, Kuala Lumpur, Malaysia. Email: janyang_ho@outlook.com

Abstract:

Metallothionein (MT) is a low molecular weight, cysteine rich stress response protein that may contribute to the pathogenesis of certain inflammatory diseases. The association between MT and periodontal diseases, however, is unclear. Therefore, the study objective is to investigate the salivary levels of MT and selected cytokines (IL-6, IL-1β and TNF-α) in systemically healthy patients with or without periodontal diseases. This cross-sectional study was conducted at a postgraduate periodontic clinic. Twenty-four patients were recruited for this pilot study. They were divided into healthy periodontium (H), gingivitis (G) or periodontitis (P) group. Unstimulated saliva samples were collected in the morning. Concentration of the proteins/cytokines of interest was determined by enzyme-linked immunosorbent assay. The average clinical attachment loss (CAL) in P group was significantly higher than both H and G groups ($p<0.05$), while the difference between H and G groups was not statistically significant ($p>0.05$) Saliva levels of IL-6, TNF-α and IL-1β were highest in the periodontitis group, followed by gingivitis and healthy group, but the difference did not reach statistical significance ($p>0.05$). CAL correlated positively with saliva levels of TNF-α ($p<0.05$). Within the limitations of this pilot study, saliva levels of IL-6, TNF-α and IL-1β were not significantly elevated in periodontitis patients, although there is a positive relationship between salivary TNF-α levels and extent of CAL.

Keywords: *Periodontitis, Metallothionein, Inflammatory cytokines*

Second prize

Title: Measurement of atherosclerosis markers in subjects with periodontitis

Presenter: Angar Soronzonbold

Authors: Angar Soronzonbold[1], Erkhbilguun Munkhkherlen[1], Uurtuya Shuumarjav[2], Oyun-Enkh Puntsag[1], Bayarchimeg Batbayar[1]

[1]Department of Periodontics and Endodontics, School of Dentistry, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia; [2]Department of Pathophysiology, School of Biomedicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia. E-mail: pmm17d011@st.mnums.edu.mn

Abstract:

Background and objectives: The prevalence of periodontitis was 71.1% in the Mongolian population in 2013. The inflammatory response due to inflammatory mediators and bacterial etiologies, and the altered lipid metabolism in patients with periodontitis suggest that infection with periodontal anaerobic bacteria may influence atherogenesis *in vitro* and *in vivo*. We aimed to explore the effect of periodontitis concerning clinical and ultrasound markers of early atherosclerosis.

Methodology: In this case-control study, a total of 30 systemically healthy adults (15 with periodontitis and 15 without periodontitis) aged over 40 years were studied. The stage and grading of periodontitis were determined by measuring the clinical attachment level (CAL) and radiographic bone loss (RBL). Traditional cardiovascular risk factors, such as body-mass index, serum levels of total cholesterol (TCH), triglycerides (TG), high-density and low-density lipoprotein (HDL, LDL) were evaluated. Carotid artery intima-media wall thickness (IMT) was assessed by ultrasonography. The individual's risk of 10-year absolute atherosclerotic vascular disease (ASCVD) was estimated by the American Heart Association Guideline on the Primary Prevention of Cardiovascular Disease.

Results and discussion: Generalized periodontitis; stage III/grade B occurred in 66.7% of subjects with periodontitis. The mean of CAL and carotid IMT were 5.02±0.9 mm, 0.084±0.01 cm versus 1.6±0.61 mm, 0.072±0.02 cm in the periodontal disease and control groups, respectively, with statistically significant differences ($p=0.001$, $p=0.037$). There were statistically significant differences in the serum levels of TCH, TG, LDL, and the 10year risk of ASCVD between the two groups ($p=0.017$). The CAL and RBL were positively associated with carotid IMT and serum cholesterol levels except for HDL, whereas the tooth loss was not associated with any markers ($p<0.05$). Compared to healthy, periodontitis subjects have 2.09 times higher odds (95% CI=1.22-3.59) to have subclinical atherosclerosis.

Conclusion: The presence of periodontitis was seen to enhance the risk of atherosclerosis.

Keywords: *Atherosclerosis, Carotid intima-media thickness, Periodontitis*

Basic research

First prize

Title: Combination of Curcumin and *Lactobacillus brevis* Exhibits Synergistic Antibacterial Effect against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*

Presenter: Rezmelia Sari

Authors: Rezmelia Sari[1], Chiara Felicita J. Seong[2], Rarinda Faridzya Fitriyanto[2], Suryono Suryono[1]

[1]Department of Periodontics, Faculty of Dentistry Universitas Gadjah Mada, Yogyakarta 55281 – Indonesia; [2] Faculty of Dentistry Universitas Gadjah Mada, Yogyakarta 55281 – Indonesia. Email: suryonodent@mail.ugm.ac.id

Abstract:

Background. Periodontitis is caused by dysbiosis of oral microflora. The dominant keystone pathogens are *Porphyromonas gingivalis* (PG) and *Aggregatibacter actinomycetemcomitans* (AA). The antibacterial properties of Curcumin are effective for these two bacteria but a combination with other synergistic substance is required to increase its bioavailability. *Lactobacillus brevis* is a probiotic that plays a role in maintaining the balance of the oral microflora by competing with pathogens.

Objectives. To analyze the synergistic effect and determine the optimal concentration of the combination of Curcumin-*Lactobacillus brevis* against PG and AA.

Methods. An in vitro well-diffusion test was conducted on Tryptic soy agar. The test group consisted of Curcumin 0.01% (Group 1; n=3), Curcumin 0.1% (Group 2; n=3), Curcumin 1% (Group 3; n=3) and Curcumin 0.01%-*Lactobacillus brevis* (Group 4; n=3), Curcumin 0.1%-*Lactobacillus brevis* (Group 5; n=3), Curcumin 1%-*Lactobacillus brevis* (Group 6; n=3). The inhibitory zone was measured by a sliding caliper. Data were analyzed using Two way Anova followed by Post-hoc.

Results and Discussion. The diameter of inhibitory zone for PG and AA in Curcumin-*Lactobacillus brevis* Group was greater than Curcumin Group ($p<0.05$). The largest diameter of inhibitory zone was showed at Group 6, namely 21.33±0.51mm (in the PG Group) and 20.67±0.70mm (in the AA Group) ($p<0.05$). The antibacterial effect of this concentration elicited the same effect for both bacteria ($p>0.05$). *Lactobacillus brevis* may induce an enzymatic conversion of Curcumin that will increase its efficacy. Conclusion. The combination of Curcumin and *Lactobacillus brevis* has a synergistic antibacterial effect. The optimal concentration is 1% Curcumin and 0.5 CFU/ml *Lactobacillus brevis*. The efficacy is similar to both PG and AA. The synergistic effect mechanism requires further research.

Keywords: *curcumin, probiotic, anti-bacterial agents, periodontal pathogen*

Second prize

Title: Nobiletin promotes BMP-induced bone formation via suppressing NF-κB signaling.

Presenter: Thira Rojasawasthien

Authors: Thira Rojasawasthien[1,2], William N. Addison[2], Takuma Matsubara[2], Michihiko Usui[1], Keisuke Nakashima[1], Shoichiro Kokabu[2]

Department of Periodontology, Kyushu Dental University Division of Molecular Signaling and Biochemistry, Kyushu Dental University Email: r19Rojasawasthien@fa.kyu-dent.ac.jp

Abstract:

Periodontitis is a chronic inflammatory disease that involves the destruction of alveolar bone. Bone morphogenetic proteins (BMPs) have a powerful osteoinductive capacity and have been used as a new adjunct to graft materials for bone regeneration. Several clinical studies demonstrate that BMPs are comparable to autogenous bone grafts in efficacy. It has also been shown that BMP's efficacy is suppressed by inflammation. Nobiletin is a polymethoxylated flavone present in the peels of citrus fruits. Previous studies have shown that nobiletin suppresses osteoclast formation and bone resorption by inhibiting NF-κB signaling and inflammatory pathways. Here in this study, we investigated the effects of nobelitin on bone formation induced by BMPs. Collagen sponge disks containing BMP-2 with or without nobiletin were implanted into the dorsal muscle pouches of C57BL/6 mice. Ectopic bones were examined by X-ray, μCT, and H&E staining. We observed that bone volume in the nobiletin group implants were higher than implants containing BMP-2 alone. Using MC3T3-E1 and primary calvarial osteoblast *in vitro*, western blotting data showed that nobiletin reduced both phosphorylated p65 and IκBα protein levels induced by TNF-α. Furthermore, nobiletin counteracted the suppressive effect of TNF-α on the induction of phosphorylated Smad 1/5 and ID-1 by BMP-4. Nobiletin also canceled the suppressive effect of TNF-α on BMP-4 induced mRNA levels of osteoblast marker genes, ALP activity and ALP staining. In conclusion, BMPs have an increased efficacy in bone formation when combined with nobiletin due to suppression of NF-κB signaling. Combining BMPs with nobiletin for adjunctive bone graft material may produce high bone regeneration potential. Keywords: Nobiletin, Bone morphogenetic proteins (BMPs), NF-κB signaling

Thai award

First prize

Title: Osteoblasts with fluorescent-tagged organelle for live cell imaging

Presenter: Phan Bhongsatiern

Authors: Phan Bhongsatiern[1,2], Tomoaki Iwayama[1], Hiromi Sakashita[1], Kiwako Tomita[1], Shuji Matsumoto[1], Mizuho Iwashita[1], Masahide Takedachi[1], Shinya Murakami[1]

[1]Department of Periodontology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan; [2]Center of Excellence in Periodontal Disease and Dental Implant, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand. E-mail: iwayama.tomoaki.dent@osaka-u.ac.jp

Abstract:

In the process of osteogenesis and cementogenesis, it is essential for osteoblasts and cementoblasts to secrete matrix vesicles (MVs). Recently, we conducted live fluorescent imaging of mineralizing osteoblasts *in vitro* and demonstrated that MVs were formed in the proximity of mitochondria and accumulated in the lysosomes. This finding suggests that mineralization process may be regulated by an interaction between lysosomes and mitochondria. To trace these and other organelles in live cell imaging and to understand the role of their interaction during mineralization, fluorescent protein (FP) labeling of organelles in osteoblasts is needed. Here, we applied the recently developed method, CRISPR-mediated insertion of exon (CRISPIE), to stably and precisely label organelles with FP in osteoblasts. CRISPIE inserts a donor FP sequence into an intron of the target gene so that non-specific insertions/deletions will be removed through RNA splicing, resulting in the nearly error-free insertion of FP. We first designed three guide RNAs with different targeting sites for Actb gene and established a CRISPIE protocol for osteoblasts. We confirmed cytoskeleton in *mEGFP-Actb* knock-in osteoblasts was labeled in green under confocal microscopy. The percentage of GFP^+ cells after transfection was quantified with flow cytometry. The recombination efficacy was 2-10%, depending on the targeting sites. The single cell-derived *mEGFP-Actb* clone was established by single cell sorting. We also generated *mRuby3-Actb* knock-in osteoblasts, where β-actin was labeled in red. Furthermore, we established *Tomm20-mEGFP, Lamp1-mEGFP,* and *Tmem192mEGFP* osteoblasts for mitochondria and lysosome labeling, respectively. These osteoblast cell lines with faithfully FP-labeled organelles hold promise as a powerful tool for dissecting the biological process of mineralization in live cells.

Keywords: *Bone mineralization, Matrix vesicle, Organelle labeling, CRISPR/Cas9*

Second prize

Title: Association between gestational diabetes mellitus and periodontitis via altered expression of microRNA-223 in gingival crevicular blood

Presenter: Teerat Sawangpanyangkura

Authors: Teerat Sawangpanyangkura[1,2], Penpan Laohapand, Dittakarn Boriboonhirunsarn, Chatkoew Boriboonhirunsarn, Nattawan Bunpeng, Kallapat Tansriratanawong[1]

[1]Department of Oral Medicine and Periodontology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand; [2]Department of Restorative Dentistry and Periodontology, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. E-mail: : kallapat.tan@mahidol.ac.th

Abstract:

Background and objectives: Periodontitis (P) has emerged as a risk factor for gestational diabetes mellitus (GDM) through several proinflammatory pathways. MicroRNA-223 (miR-223) is potentially involved in inflammatory diseases, including GDM and P. Moreover, we first described a procedure for purifying miR-223 from gingival crevicular blood (GCB). The objectives of present study were to determine the association between P and GDM, and the expression of miR223 in GCB compared to those in peripheral blood (PB).

Methodology: The study included 128 pregnant women: 64 with and 64 without GDM. All participants were examined for clinical parameters of GDM and periodontal conditions. 40 participants were then randomly selected and allocated into 4 groups according to GDM and P status. GCB and PB were collected to assess expression of miR-223 and its related genes by quantitative real-time polymerase chain reaction.

Results: P was significantly more prevalent in GDM group than healthy group with an odds ratio (OR) of 2.59 (1.19 to 5.65) (P = 0.017) after adjusting for potential confounding factors. The GDM with P group demonstrated the highest miR-223 expression levels among the 4 groups in GCB. A significant difference was found between GDM with P and GDM without P group (P = 0.04). In contrast, the GDM with P showed the lowest miR-223 expression level in PB among the 4 groups. Moreover, ICAM-1 and IL-1β mRNA expression exhibited the opposite trend of miRNA-223, indicating that miRNA-223 might regulate the mRNA function of those genes by epigenetic events.

Conclusion: the altered expression of miR-223 in GCB and PB of pregnant women with GDM and P suggested a promising role of miR-223 in the association between GDM and periodontitis.

Keywords: *Gestational diabetes mellitus; miR-223; gingival crevicular blood; periodontitis.*