DETOXIFICATION OF TITANIUM IMPLANT SURFACES: EVALUATION OF SURFACE MORPHOLOGY AND PRE-OSTEOBLAST CELL COMPATIBILITY

by

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Dedicated to my family, friends and mentors for their endless support and motivation.

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THESIS

Presented to the Faculty of

The University of Texas at Dallas

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN

BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT DALLAS

December 2016

ACKNOWLEDGMENTS

This research study was made possible by an incredible group of individuals. I would like to express by sincere gratitude and appreciate for all those involved. Being a student at The University of Texas at Dallas gave me the opportunity to gain knowledge and hands on experience in research and development in the Bioengineering field of study. Dr. Danieli C. Rodrigues gave me a chance to take my educational career to the next step by giving me the opportunity to pursue my research goals in The Biomaterials for Osseointegration and Novel Engineering (BONE) Lab. From the beginning, she has been a great role model and mentor. She gave me unconditional support and motivation throughout this journey and for that I am very thankful.

I would like to extend my appreciation and gratitude to our collaborators Dr. Pilar Valderamma and Dr. Thomas G. Wilson Jr. for providing clinical expertise and support for this project. I would also like to thank Dr. Kelli Palmer for allowing me to use her lab resources and also giving me guidance and expertise to carry out microbial studies, the appreciation also extends to her lab members for their help. Thanks to Dr. Heather Hayenga and her lab members for their support and for letting me use the cell culture resources to carry out my cell studies.

Thank you to my committee members Dr. Kelli Palmer and Dr. Taylor Ware for being so patient and supportive, thank you for being a part of this journey.

Not only did I gain extensive knowledge and skillset while working in the BONE lab, I also made many good friends who were all helped me complete this project. Thank you to Izabelle Gindri, Shant Aghyarian, Lucas Rodrigues and Sathyanarayanan Sridhar for giving me such a warm welcome into the lab, seeing you all work gave me so much confidence and ambition to pursue my aspirations. I would like to acknowledge and thank Sathyanarayanan Sridhar for always being there whenever I had doubts or questions. His advice, suggestions and guidance drove me to the right path. Thank you to Lucas Rodriguez and Juliana Saba for spending so much of your time and effort to train me for cell studies, I would not have been able to do it without your guidance. Thank you to Danyal Siddiqui for his unconditional help; his genuine devotion to research motivated me to be a better student and researcher. My sincere gratitude extends to Pavan Preet Kaur who has been a constant support; her pep-talks and motivational texts really drove me to the finish line. I would also like to express my appreciation to Sutton Wheelis who shared her knowledge and expertise on all things dental implants and detoxification. Thank you to Vidyalakshmi Jayaraman, Gina Quiram, Frederick Wang and Jason Chang for your help and support.

Lastly, I would like to thank my parents and family for their continuous guidance and encouragement to accomplish my dreams and goals. Their love and support gave me the determination and strength to always aim higher. This would not have been possible without you.

November 2016

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Publication No. _____

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The University of Texas at Dallas, 2016

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Success of a dental implant is primarily assessed by its surface condition and its capability to biologically integrate with surrounding soft and hard tissues. When the surface of an implant is compromised by bacterial adhesion, it can result in development of peri-implantitis and ultimately implant loss. One of the primary etiological factors resulting in peri-implantitis is the formation of a biofilm created by adhesion of bacteria on implant surfaces. Peri-implantitis is a site-specific disease that causes bone loss and inflammation around a functional implant. Clinicians commonly use a combination of mechanical debridement/detoxification methods with acidic chemicals to remove adhered biofilm. It is hypothesized that acidic conditions caused by these detoxification chemicals, in addition to mechanical abrasion, can lead to surface changes including pitting, corrosion and discoloration, which can affect the growth of bone-forming cells. The study's main goal was to evaluate changes in surface morphology of titanium after bacterial adhesion and detoxification procedures. In addition, proliferation and differentiation of bone-forming cells were

analyzed after exposure to bacterial adhesion and detoxification procedures on implant surfaces to infer about re-osseointegration post-treatment.

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CHAPTER 1

INTRODUCTION

Over the years, dental implants have been transformed by innovative designs, material combinations and surface coatings, which are all aimed at improving performance and offering long-term restoration and aesthetic appeal. Modern dental implants offer faster healing time, better function and comfort to patients [1]. It has been estimated that one in four Americans over the age of 74 have lost all their teeth, and 69% of Americans aged 35-44 have at least one missing tooth due to the rise in periodontal diseases in the aging population [2]. Dental implants can be used to replace one, multiple or all missing teeth due to injury, decay or defects [3]. More than 500,000 implants are placed every year and this number is expected to rise in the U.S [3]. The American Academy of Implant Dentistry (AAID) has estimated that the U.S and European markets for dental implants will reach $4.2 billion by the year 2022 [3]. Currently, over 3 million people have implants with success rates ranging from 90-95% [2,4].

The general method to place a dental implant can be seen in Figure 1.1. This procedure involves removing the natural tooth followed by drilling a hole into the jaw bone for placing the implant. Next a screw cover is placed in the hole, and clinician waits a couple of weeks before placing the abutment. After the abutment is attached to the implant, a ceramic crown is attached [5].

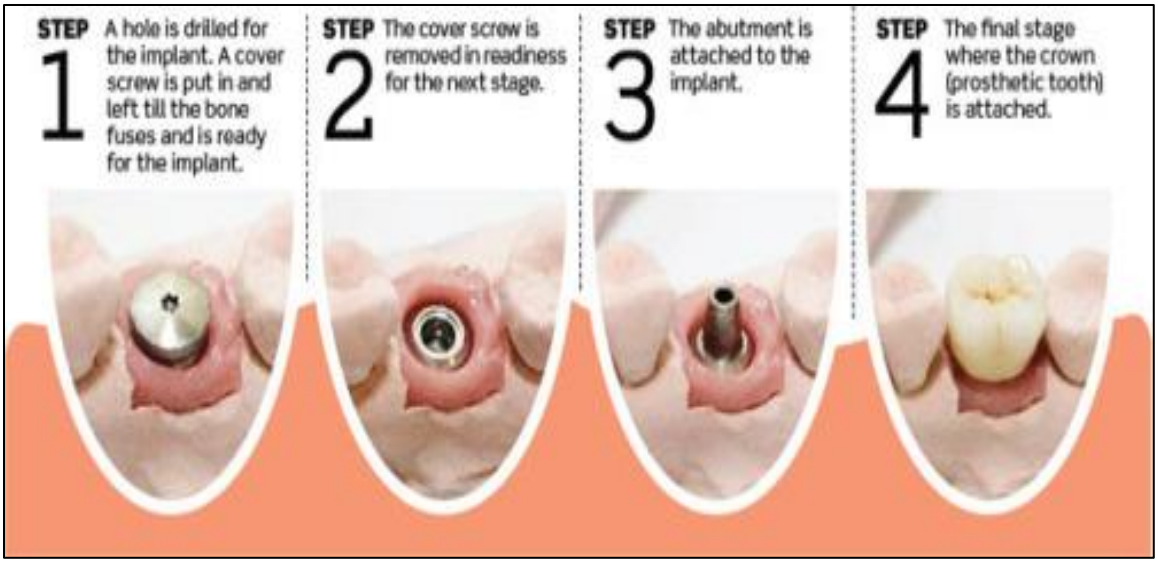

Figure 1.1 General steps involved in placement of a dental implant. Source: (http://randallwooddental.com/blog/?cat=4).

One of the most important parameters for success of a dental implant is surface interaction and integration with surrounding hard and soft tissues to achieve mechanical support and stability. Osseointegration is defined as the functional and structural connection between ordered, living bone and the surface of a load-bearing implant [6]. Osseointegration is recognized as a major factor influencing long-term clinical success [6]. In addition to osseointegration, biocompatibility, mechanical strength and corrosion resistance are some of the attributes that are considered critical in the design of a successful dental implant [7]. One of the most commonly used material in the design of dental implants is commercially pure titanium (cpTi). This is because this material exhibits excellent biocompatibility, mechanical strength and high corrosion resistance. These excellent properties result from the material's ability to spontaneously form a passive oxide layer

(TiO_2) in the presence of oxygen. This layer acts as a protective barrier to continued oxidation and metal dissolution [8, 9, 10].

Even with a success rate of 90-95% [4], dental implants do fail *in vivo*. Failures are typically categorized into early and late stage failures depending on the period following implantation and level of osseointegration between implant and surrounding bone [11]. Early complications show up before an implant has osseointegrated, while late failures tend to happen after osseointegration has been established [11, 12]. Within these two categories, there can be biological or mechanical reasons leading to failure [11].

This work presents a comprehensive study of the effects of a specific treatment method known as detoxification/debridement on titanium dental implant surfaces. This treatment method is commonly applied to peri-implantitis-infected dental implants. There are two main categories of this biological disease. Peri-mucositis and peri-implantitis are pathological periodontal diseases that may lead to implant loss if not properly treated. Peri-mucositis is the inflammation of soft tissues neighboring the implant, which is easily treated and reversible [13]. However, if this condition is not properly treated it can progress into peri-implantitis. Peri-implantitis causes irreversible inflammatory lesions of the mucosa in addition to loss of osseointegration in the supporting bone, which can be challenging to cure as shown in Figure 1.2 [14]. Both diseases are communicable and are triggered by bacteria forming a biofilm on the implant surface [7].

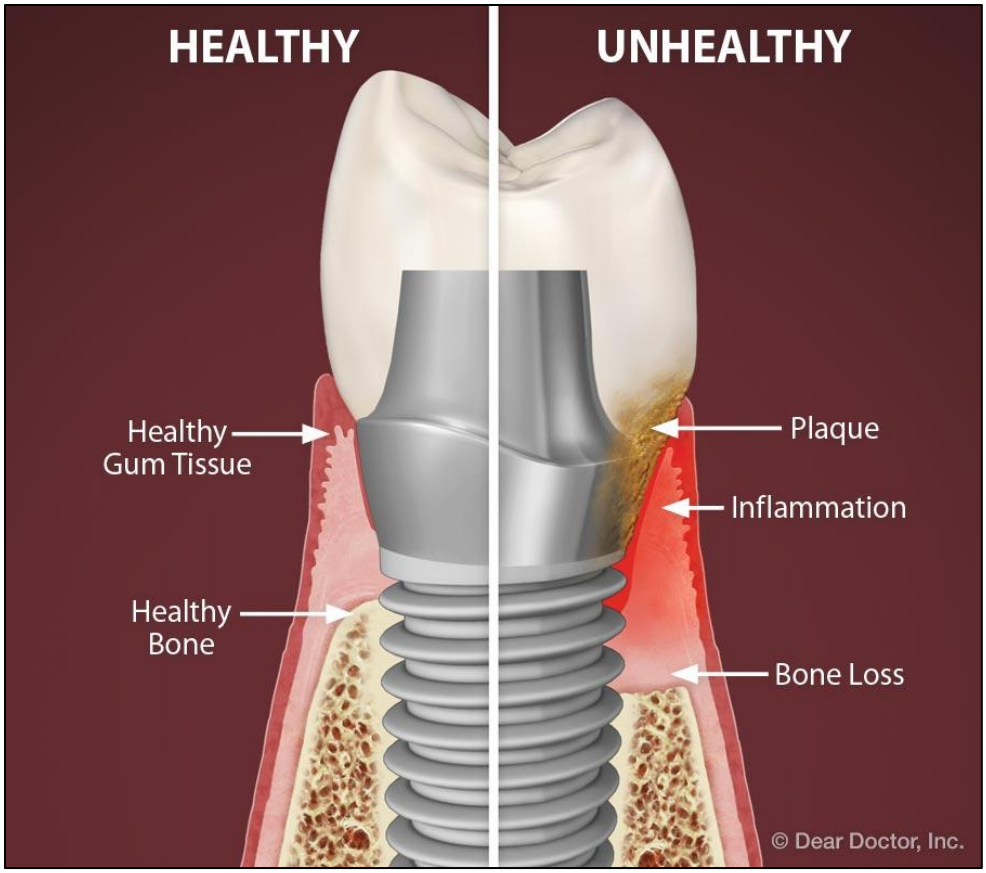

Figure 1.2 Difference between a healthy implant and unhealthy implant. Inflammation and bone loss are present in peri-implantitis infected dental implants. Source: (http://www.deardoctor.com/articles/peri-implantitis-can-cause-implant-failure/).

Peri-implantitis is a disease triggered mainly by the synergistic effects of four factors: lesions of peri-implant attachment [15], bacterial contamination of implant surfaces [16, 17], excessive mechanical loading [15] and corrosion [15]. Of these factors, Mohyi *et al*. suggested that corrosion may be more than a triggering factor and is considered as a phenomenon underlining osseointegration [18], subsequently proposing that peri-implantitis is an osseointegration pathology [15]. Treatment for peri-implantitis requires a specific and localized approach to stop each triggering factor. It must provide regeneration of supporting lost bone to re-establish

mechanical support while simultaneously confronting bacterial contamination on implant surface [15]. When peri-implantitis is diagnosed, the clinician has two options: 1) try to recover the infected implant; or 2) completely remove the implant, typically the first option is chosen. Treatment methods currently used by clinicians employ chemical, mechanical and laser approaches to remove adhered biofilm [19-22]. For example, antimicrobial therapy [23], regenerative procedures [24, 25], CO_2 laser [26], air powder abrasive [15] and micro-abrasion with chemicals [15] are commonly employed. Most of these treatments focus primarily on decontamination of the implant surface instead of trying to recover the de-osseointegrated interface between supporting bone and implant surface. A commonly accepted method of treatment is by debridement/detoxification of implant surfaces with chemicals to manually remove the biofilm [27]. Frequently used chemicals include: citric acid, tetracycline, saline, chlorhexidine, hydrogen peroxide, tetracycline, and doxycycline [28-30, 27]. Although, detoxification methods are commonly employed in the treatment of infected implants, literature reports have shown both negative and positive results for this treatment method.

An *in vivo* human study done by Leonhardt *et al*. [31], on peri-implantitis-infected Branemark implants, showed only a 58% success rate after a 5-year follow up to a combination treatment. In this study, the combination treatment included detoxification with 10% hydrogen peroxide, saline solution wash followed by the use of 0.2% chlorhexidine mouthwash and systemic antibiotic treatment. Another human study conducted by Heitz-Mayfield *et al.* [32] in 2012 analyzed a different treatment method on machined Ti-Unite, TiO-blast, and plasma-sprayed implants. They used carbon fiber curettes with saline irrigation to scrape off the biofilm. Subsequently followed by prescription of antibiotics for 7 weeks along with 0.02% chlorhexidine mouthwash for 4 weeks

to completely detoxify the implant surface. After a 12-month follow up to this treatment, a 100% survival rate for the treated implants was observed.

These two studies are a few of many that have been published in this field. There is a broad spectrum of detoxification results reported due to variations in treatment method as well as variations in type of dental implants investigated. In addition, there is a lack of understanding of how exactly bacterial adhesion and detoxification change the morphology of the surface of dental implants and, synergistically, how these two processes impact growth of bone-forming cells.

The goal of this study is to design and develop an *in vitro* testing model to study the combined effects of bacterial adhesion on dental implant surfaces that will then be detoxified with chemicals and subsequently exposed to bone-forming cells. This sequence of events will enable investigation of cell behavior in response to treatment. Results of this study will help elucidate how bacterial adhesion and detoxification affects implant surfaces, in addition to how bone-forming cells respond to detoxification with various chemicals.

This thesis is divided into chapters and they are explained as follows. Chapter 2 gives insight into the background information about the general problems associated with dental implants, specifically discussing peri-implantitis and how it affects implant surfaces. Causes, diagnosis, treatment methods, motivation and clinical importance are discussed as well. Chapter 3 states the goals, tasks, rationale and hypothesis for the study. Chapter 4 gives the results and discussion of the experiments executed in this work and it is structured in manuscript form. It presents an introduction of the detoxification of titanium dental implants with chemical and mechanical means, then stating materials and methods involved in the study, results, discussion and conclusion

follows. Chapter 5 gives a brief summary of the results obtained and the overall findings. Lastly, Chapter 6 offers ideas for future work as a continuation of this project, which will help to better understand the effects of detoxification method on the long-term success of dental implants.

CHAPTER 2

BACKGROUND

This chapter covers the key characteristics of dental implants and general clinical problems associated with them. Peri-implantitis will be discussed as well in addition to available treatment options and their advantages and disadvantages. Lastly, motivation for the research study, goals and clinical importance will be stated.

2.1 DENTAL IMPLANT SUCCESS AND DESIGN CONSIDERATIONS

Researchers and clinicians are on a long-pursuit to increase the lifespan of dental implants *in vivo.* Today, the dental implant technology has come a long-way since the discovery of a 4000-year-old bamboo dental implant in China and the sea 'shells' placed as implant by the Mayans [33] as illustrated in Figure 2.1

Figure 2.1 Evidence of world's first dental implant dating back to 600AD found in Honduras. Source: (https://lasvegasdentalimplants.wordpress.com/).

Novel materials, design considerations and surface coatings are being researched and developed to give patients the very best dental care. But, like other implant systems, insertion of a foreign object in the body triggers a complex response because of the inter-relation between different systems of the human body. Therefore, when trying to increase the lifespan of a dental implant *in vivo*, knowing the various aspects that contribute to this response is crucial. Research shows that success of a dental implant depends on the combination of many factors as summarized in Figure 2.2.

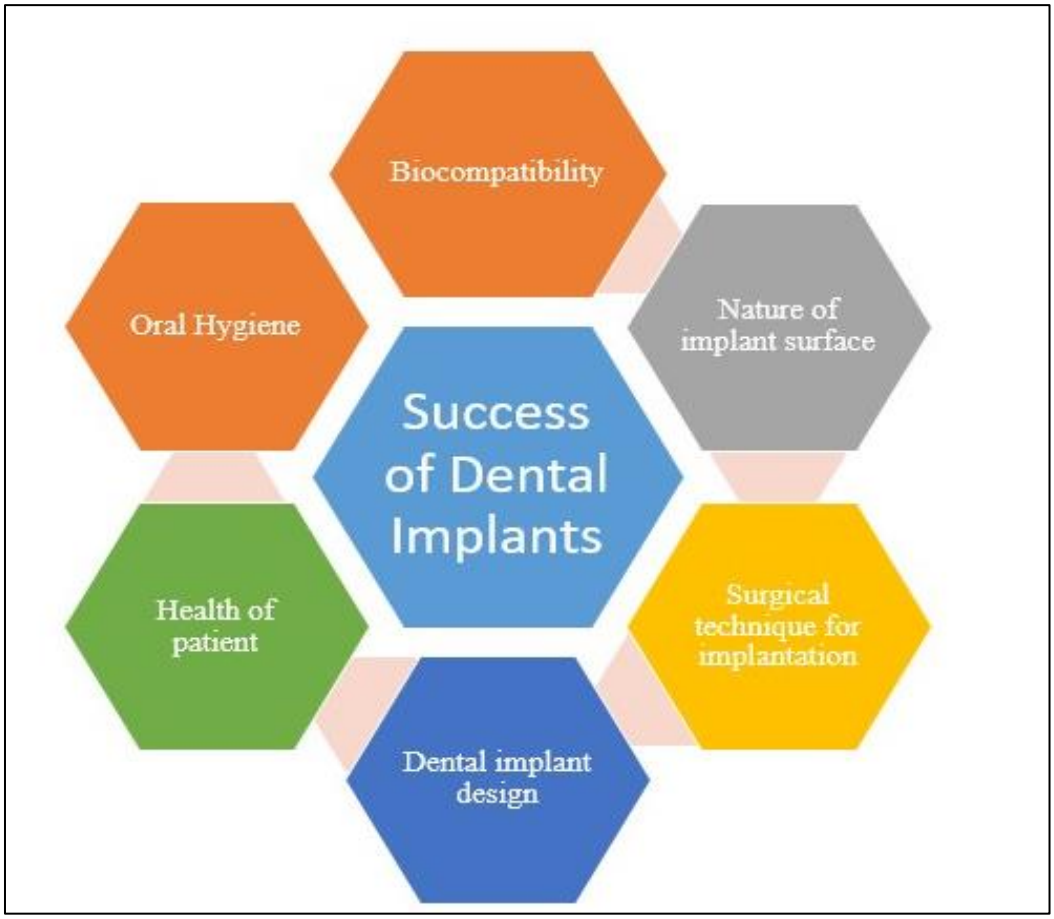

Figure 2.2 Factors that affect long-term success of a dental implant *in vivo.*

One of the most important design factors for any implant system is biocompatibility [34]. Biocompatibility is simply a characteristic of a material that allows the material to not elicit a negative response upon interaction with surrounding tissues. This is the backbone for long-term success.

Along with biocompatibility, osseointegration is equally an essential requirement for dental implants. If no osseointegration is achieved, an implant will not survive for long *in vivo* because of the lack of mechanical support. Some design considerations that affect osseointegration include implant material composition, implant design, biomechanical factors, surface characteristics, and patient bone health [35-37].

Materials currently used to design and develop dental implants include: metals, carbons, polymers, ceramics and a combination of them [34]. Of these, commercially pure titanium (cpTi) and its alloys (Ti6AL4V) have conquered majority use in the dental implant industry due to their biocompatibility, corrosion resistance, durability [34], and mechanical properties [38]. In addition, titanium has a naturally forming oxide layer [39], which provides protection against corrosion and encourages osseointegration of bone onto implant surfaces by providing a platform for bone matrix to grow. Other metals such as gold, zirconium, and hydroxyapatite have been used previously as components of dental implants.

Surface characteristics play a crucial role in the success of dental implants. When the implant is placed *in vivo,* blood cells, proteins, sugars, lipids, and surrounding tissue cells get absorbed on the surface and information gets exchanged resulting in activation of genes [34]. This early process determines acceptance or rejection of an implant [7]. Chemical and physical properties of the

implant surface regulate quality and speed of osseointegration [34]. Thus, a lot of research has gone into changing the surface characteristics such as morphology, roughness, energy and thickness of surface coatings to decrease adhesion of bacteria and increase adhesion of bone-forming cells on implant surfaces [7]. Surface features are altered by machining, plasma spraying, machine grit-blasting, acid-etching, anodization and laser treatments [34]. These techniques are known to change the surface morphology of dental implant creating pores and venues for better tissue integration. The different surface types of dental implants are illustrated in Figure 2.3.

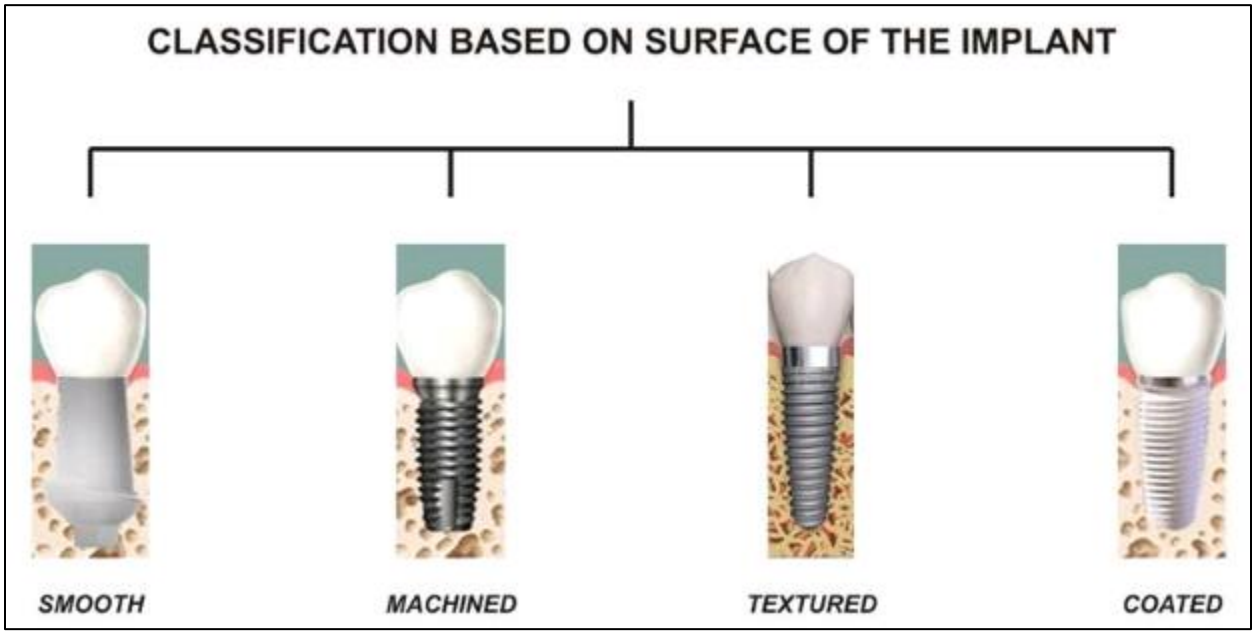

Figure 2.3 Classification of implant type based on surface characteristics. Source: (http://www.dentalimplantcostguide.com/types-of-dental-implants/).

In addition to implant material and surface characteristics, patient health is evaluated before the placement of an implant. Clinical studies have revealed bone quality and general health of patients as being factors that influence the life spam of an implant [40]. Smoking, diabetes, osteoporosis,

microbial and immune-inflammatory factors, and other factors increase chances of implant failure [40]. Therefore, before a patient receives a dental implant, they are made aware of these health issues and advised to improve their health habits and oral hygiene to increase the chance of implant success.

With all this technology available for application in dental devices, dental implant companies manufacture implants with different materials, design and surface treatments. Table 2.1 summarizes examples of different types of dental implants that are in current use [41].

Recently, the use of zirconia (ZrO_2) for dental implant design is on the rise because of its inert properties and minimal ion release [48]. Similarly, zirconia offers reduced inflammation and bone resorption making this material a much better option than metals such as titanium.

The advantages zirconia offers influenced many companies to start exploring this material. For example, Straumann, which is one of the leading manufacturers of dental implants recently introduced a new implant system in the market (Roxolid®). This implant system is made of an alloy of titanium and zirconium (TiZr), which is believed to combine the best properties of both materials in a unique alloy. Roxolid® is marketed as a premium material possessing better mechanical strength and resistance to corrosion in biological fluids [49-51]. Studies done by Ikarashi *et al*. [52] revealed that TiZr alloy shows better biocompatibility and mechanical properties than cpTi [52].

Table 2.1 Various types of dental implant systems in current use and studies evaluating their performance.

Implant Type	Design & Material	Research
Blade-vent implants	CrNiVa-alloy, titanium alloy, aluminam oxides, vitreous carbon	Natiella *et al*. inserted 149 blades in a monkey model over a 0-36 month follow up. 10% of the implants failed. [42] Cranin, Rabkin, and Garfinkel placed 952 blades in 458 patients. After a 5-year follow up, success rate was 55% [43]
Tübingen aluminum ceramic implant	Irregular conical cylinder with surface lacunae made of Aluminum oxide	There is convincing evidence that aluminum oxide Tübingen implants become anchored in bone without intervening soft tissue layers. [44]
TCP-implant	Cylindrical, titanium implant coated with tri- and tetracalciumphosphate	Animal studies showed direct bone contact with the TCP-implant [45]
ITI hollow-cylinder implant	Plasma-sprayed surface made of titanium	A 90.0% success rate was found after a 1 year follow up with 11 implants as reported by Ledermann *et al*. [46, 47]

2.2 CURRENT PROBLEMS ASSOCIATED WITH DENTAL IMPLANTS

Even with a high success rate, 5-10% of implants have been shown in the literature to fail [4] as discussed in previous sections. While severe complications are rare, dental implant surgery is a complicated procedure and problems can arise at any period of the process [53]. A dental implant is considered successful if after the first year the patient doesn't suffer from pain, implant loosening, or 1 mm or less bone loss [41].

There are numerous complications and failures that may occur, some of which are associated with the surgical procedure, bone loss, peri-implantitis, mechanical or esthetic issues [54]. However, implant complication and implant failure are not the same; hence, it is important the clinician is able to distinguish between them to provide better care and treatment. Implant complication involves failure of a specific component of the implant, for example, a loose screw which can be repaired or replaced. Implant failure mainly is distinguished by loss of osseointegration [34].

Once the implant is placed, the process of healing takes approximately 3-6 months. During this period, titanium implant osseointegrates with the surrounding bone creating a mechanically stable and strong support. This initial 3-6-month period is critical because it dictates long-term success of an implant, but even after osseointegration, there are chances of implant failures. Poor hygiene can play a crucial role in implant success.

Implant failures are mainly classified into early and late-stage failures. Bacteria/bacterial biofilm has been assumed as the primary reason for both early- and late- stage complications. Early stage failure occurs before attachment of prosthetic components primarily due to failure to establish

osseointegration [55, 56]. Early stage failure is associated with early colonizers such as *Streptococcus* and *Actinomyces* [57, 58]. These colonizers are known to initiate biofilm formation, which fosters growth of late colonizing anaerobic pathogens such as *Porphymonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* etc., which are associated with periodontitis [59, 60]. General factors that contribute to early implant failure include poor bone quality and quantity, poor bone healing and general health conditions, smoking, clinical signs of infection, premature loading, post-insertion pain, and lack of primary stability [61].

Late stage complications occur after osseointegration is established between implant and surrounding bone. This type of failure is also associated with bacteria-induced marginal bone loss (peri-implantitis) and excessive occlusal stresses [37]. Factors that contribute to late implant failures include excessive loading, peri-implantitis and inadequate prosthetic construction [61]. Excessive loading usually occurs when the load applied is beyond the level the bone can withstand. Late failures can also occur if the implant is improperly fitted into the bone leading to fractures or loose screws.

2.2.1 Peri-implantitis

Recently, a rising number of implant failures has been reported due to peri-implantitis, which is a clinical condition characterized by inflammation and continued loss of integrated bone around an implant [34, 38]. A study reported that 28%-56% of patients who receive an implant suffer from this severe clinical condition [39].

As soon as the implant is anchored in the jaw bone *in vivo*, the surface is immediately exposed to the microbial environment of the oral cavity and gets colonized by several micro-organisms [61]. In addition to microorganisms' polysaccharides, proteins and nucleic acids [63] all come in contact with the implant's surface. These micro-organisms can cause periodontal diseases; bacteria commonly associated with peri-implantitis are gram-negative anaerobes such as *Prevotella intermedia, porhyromonas gingivalis, actinobacillus actinomycetemcomitans, bacteroides forsythus, Treponema denticola, prevotella nigrescens, peptostreptococcus micros and fusobacerium nucleatum* [62-64]. In addition to these, *Streptococcus sanguinis* [65-68] and *staphylococcus aureus* [13] are known as the pioneer colonizers in oral biofilms and identified to bind to hard surfaces such as implant surfaces [69, 27].

The first step involves adhesion of salivary proteins, mucins and glycoproteins onto an implant surface, which form a layer commonly known as pellicle; this occurs within minutes of the implantation. Next, bacteria adhere to the formed pellicle layer and start proliferating in numbers within a few hours, forming micro-colonies. Subsequently, more bacteria start attaching to the first layer of bacteria creating a multi-layer biofilm as illustrated in Figure 2.4 [63].

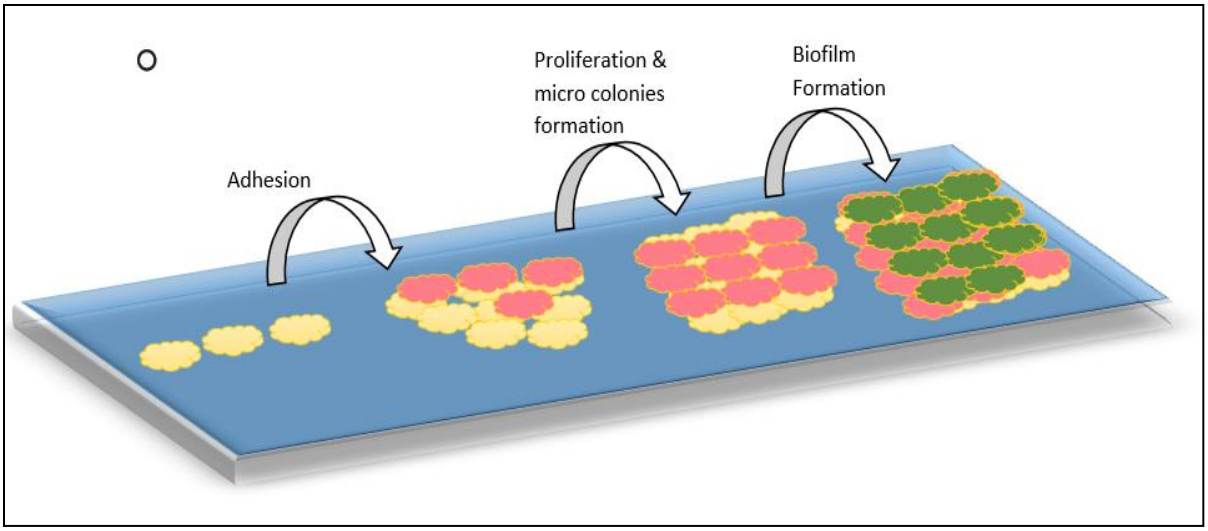

Figure 2.4 An image illustrating the formation and development of bacterial biofilm.

This pathogenic biofilm results in development of peri-implant mucosa and peri-implantitis [65-68]. As discussed earlier in the summary, peri-implant mucosa is the reversible inflammatory disease that effects only the soft tissue around an implant [13]. Peri-implant mucositis is identified by the presence of bleeding on probing with no evidence of radiographic loss of bone around an implant [70]. On the other hand, peri-implantitis is a site specific inflammation of soft tissues and the partial destruction of supporting bone around an implant [69]. Peri-implantitis is further classified into different categories depending on the extend of bone loss seen. Georgios E. Romanos *et al.* [71] summarized the classification of peri-implantitis as seen in Figure 2.5.

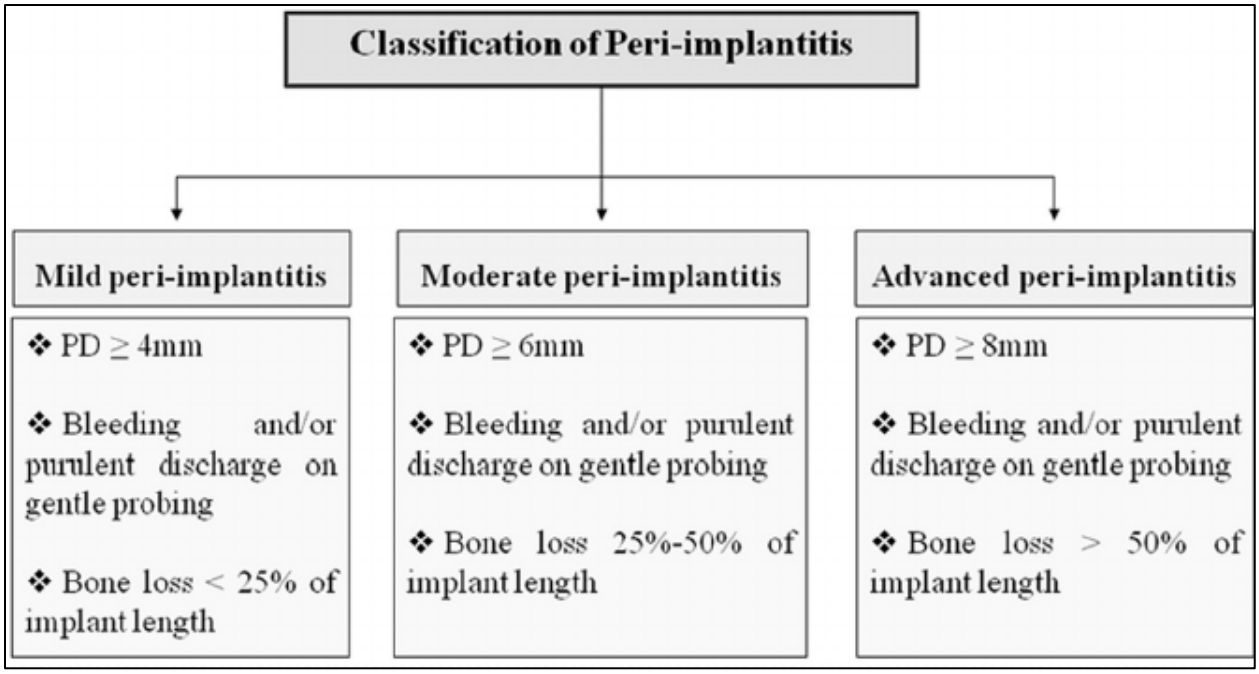

Figure 2.5 Classification of peri-implantitis summarized by Georgios E. Romanos *et al.* [71]. (PD: Pocket depth).

Early detection of peri-implantitis is challenging, but the defect can be identified as a saucer-shaped area with bone destruction due to peri-implant pocket formation [72]. Often differentiating between peri-implantitis mucosa and peri-implantitis itself is quite difficult. Consequently, it is often detected only after patient suffers from its symptoms. Some symptoms include bleeding of the gums during brushing, swelling, bad breath or loosening of the implant.

Although treatment for peri-mucositis can be done by nonsurgical methods, it has not been proven to be the best practice. Antibiotic therapy and mouth rinses are typical nonsurgical remedies. There is a risk of bacteria recolonization, which can progress into peri-implantitis. Therefore, the patient

is usually monitored for several weeks during nonsurgical treatments [73]. And if after monitoring, infection persists, clinicians usually carry out surgical methods of treatment.

2.3 TREATMENT METODS FOR PERI-IMPLANTITIS INFECTED IMPLANTS

When faced with a peri-implantitis infected dental implant, a clinician has two options: 1) remove the implant or 2) treat the infected implant, in which usually the latter is done. There are mechanical, chemical, lasers and a combination of treatment methods for implant surface detoxification as summarized in Figure 2.6.

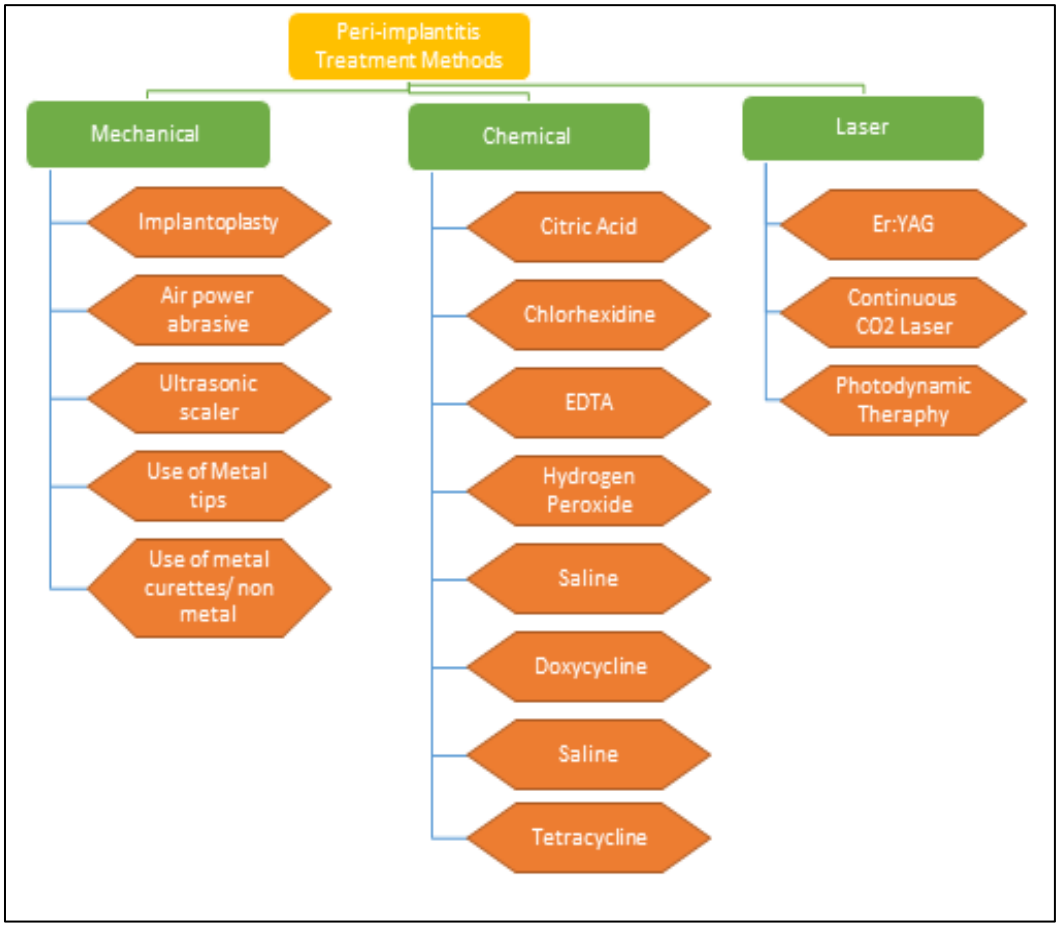

Figure 2.6 A schematic representation of the different treatment methods available for peri-implantitis.

2.3.1 Mechanical Methods of Detoxification

Mechanical detoxification methods include implantoplasty, air powder abrasive, ultrasonic scaler, use of metal tips, metal curettes or nonmetal curettes [27]. Implantoplasty is a technique recommended by Lang *et al*. [74], which intends to mechanically scrap adhered bacteria from the surface and smoothen it to prevent further plaque adhesion on surfaces [75]. A three-year human study revealed implants had a higher survival rate and prevented marginal bone loss after implantoplasty treatment [76]. One negative aspect of this procedure is that it increases post-operative recession of tissues and exposes the abutment; thereby, increasing food impaction [27]. In addition, this procedure produces heat and can damage implant surfaces by introducing defects, delamination of top layers, and cracks, which can result in weakening of the metal structure.

On the other hand, air power abrasive technique uses a stream of compressed air with sodium bicarbonate or amino acid glycine power and water to remove adhered biofilm. Cleaning efficiency of this method has been proven to be 100% *in vitro* [77], but the effect it has on osseointegration is currently unknown.

The use of curettes has been shown to reduce roughness of implant surfaces. There are metal as well as non-metal curettes commercially available. A study has shown the use of metal curettes to be an efficient *S. sanguinis* disrupter [78]. Non-metal curettes are made of carbon, resin-reinforced and resin-un-reinforced as well as plastic. Non-metal curettes are not commonly used because they have been shown to be inefficient at removing bacteria [79].

2.3.2 Chemical Methods of Detoxification

Along with mechanical methods, clinicians usually employ chemicals for debridement to eliminate adhered biofilm. Some commonly used chemicals include: citric acid, chlorhexidine, ethylene diamine tetra-acetic acid, hydrogen peroxide, saline, and tetracycline [27]. Although there is not a properly established or recommended chemical for treatment of peri-implantitis-infected implants, there are a few chemicals that offer better treatment than the other.

Citric acid (CA) is an extensively used chemical and comprehensively studied for the treatment of peri-implantitis. One detail that has not been established to date is what concentration is most effective and for what period of time should the chemical be applied for complete and efficient bacteria removal. An *in vitro* study showed a significant reduction of *E. coli* LPS on titanium alloy when burnished for 1 minute with a CA soaked cotton pellet [80]. When cytotoxicity of CA was studied, a significant decrease in cell proliferation was reported, but no cytotoxicity was observed at 4% and 10% concentration of CA [81].

Chlorhexidine (CHX) has also been broadly employed for peri-implantitis treatment due to its bactericidal and antiseptic properties. Many studies have demonstrated chlorhexidine (0.12%) to reduce the amount of *Porphyromonas gingivalis* (a pioneer periodontal disease bacteria) by 92.9% on titanium plasma sprayed surfaces [82]. An *in vitro* study evaluated osteoblast viability after treatment with chlorhexidine (CHX). These results showed the quantification in apoptotic and necrotic cell death that occurs due to variation in chlorhexidine-dose [83], another study revealed CHX inhibits cell proliferation [84].

Saline has been proved to provide decontamination as well as re-osseointegration for dental implants. Human studies showed that the combined effect of mechanical scrubbing with saline soaked curettes on peri-implantitis-infected implants resulted in stable implantation for up to 24 months [85, 86]. Hydrogen peroxide (HP) was also evaluated for its potential to eliminate *Candida albicans* and *S. sanguinis*, but results showed that this treatment was only effective against *C. albicans* [87]. And finally, EDTA had been mainly used because of its neutral pH and common use for removal of smear layer before application of biomimetic material for regeneration [27].

2.3.3 Lasers for Detoxification

A commonly used laser for decontamination is the Eribium-Doped: Yttrium, Aluminum Garnet (Er:YAG) laser. An *in vitro* study comparing Er:YAG laser to a plastic curette method for detoxification showed the laser was better at removing early biofilm, but it did not reinstate biocompatibility to the surface [88]. The other laser available for detoxification is the continuous CO_2 laser, but this laser is known to burn the bacteria on the surface instead of actually removing the biofilm, thus this treatment option is not commonly used [27].

From the above mentioned peri-implantitis treatment methods, there is no properly established treatment that has been 100% successful. Instead there are numerous methods, from which the clinician can choose from, and when used hoping it will be efficient in treating peri-implantitis-infected implants. Hence, these methods are typically combined with other treatments to increase efficiency. Sometimes, clinicians use laser treatment along with prescribing the use of an antibiotic for a period of time, or they will use mechanical debridement with an antibiotic and then irrigate with a chemical.

A common combination is the use of mechanical debridement with curettes or cotton pellet soaked in acidic chemicals to kill and remove bacterial biofilm. The effects of this method on titanium surfaces is unknown. As mentioned, most clinical studies base their conclusions on qualitative observation of tissue health around implants and radiographic observations of osseointegration. Thus in this study, growth of bone-forming cells on titanium surfaces after being subjected to bacterial adhesion and detoxification will be analyzed to conclude on the consequences of this treatment.

Prevalence of peri-implantitis is increasing as the number of dental implants placed increases and with so many options for treatment, it is risky to proceed with a particular one without knowledge of its possible effects on the long-term success of the implant. Thus, investigation of the effects of these methods on implant surfaces and how it stimulates or impedes re-osseointegration is an important question to be addressed. In-addition to understanding how treatment methods can affect implant surfaces, examining the effects bacterial biofilms have on implant surface morphology is equally important. Only a few studies have examined the effects of bacterial adhesion on implant surfaces. Knowing how bacteria impacts the surface of an implant can contribute to the development of better materials and surface treatments that resist or hinder absorption of these microbes on the surface.

Extensive research has been clinically done in patients to investigate the effects of treatment methods on titanium and its alloys [89-92]. However, there is a lack of information regarding how synergistic effects of bacterial adhesion, detoxification and cell compatibility change the surface

of pure titanium and how this entire process can affect proliferation and differentiation of bone-forming cells.

The goal of this study is to understand the synergistic influence of bacteria, biofilm adhesion and detoxification on the morphology of titanium surface and how this affects bone cell adhesion. Bacterial biofilm has been discussed to create areas of oxygen depletion on the surface of an implant, creating a low pH environment, which can result in crevice corrosion [93]. This can result in metal ion depletion, discoloration and or pitting attack of the metal substrate [94]. Additionally, bacteria are capable of releasing acidic metabolites such as lactic acid onto the surface of the implant, creating similar conditions of low pH and acidic environment. *Streptococcus species* is a known lactic acid metabolite producer [95], and these species play a significant role in formation of biofilms as previously stated.

In this study an *in vitro* technique will be developed to allow for examination of the titanium surface after: (1) exposure to bacteria, (2) detoxification with acidic chemicals, (3) direct contact with host cells to investigate growth of bone-forming cells post-detoxification. Investigation of the material in these tasks will provide information about surface morphology changes after exposure to acidic environments, and how this can impact cell proliferation and differentiation to infer upon possibility of re-osseointegration. Completion of these tasks will provide possibility of better understanding how each chemical changes the surface of titanium and how cells interact and integrate on this material after detoxification procedures.

In summary, the tasks of this study will be accomplished by exposing pure titanium (cpTi) samples to an *in vitro* environment clinically relevant to the oral cavity of a peri-implantitis infected dental

implants. Bacteria will adhere to the sample surface developing a biofilm, these will be visualized using optical microscopy (OM) and scanning electron microscopy (SEM) to identify signs of corrosion. Next, detoxification/debridement will be performed on these samples to remove adhered bacteria (similar to what a clinician does at the office). Chemicals commonly used in the clinics were selected for simulated detoxification. Likewise, optical microscopy and scanning electron microscopy will be used to visualize any morphological changes to the surface post-detoxification. Lastly, these specimens will be placed in direct contact with pre-osteoblast cells to assess compatibility and differentiation of cells after detoxification has been done. Pre-osteoblasts are the cells that create a matrix on an implant surface which ultimately form bone; therefore, this evaluation will contribute to the understanding of how bone-forming cells are able to respond to surfaces that have been exposed to detoxification.

CHAPTER 3

GOALS AND HYPOTHESIS

GOAL 1: To contaminate the surface of titanium with peri-implantitis-inducing bacterial species.

- Develop a contamination protocol
- Determine which bacterial strains to be used in mixed bacterial strain
- Acquire protocol for bacterial culture media preparation
- Decide optimal incubation period for titanium specimens in bacterial culture media
- Develop protocol for preparing bacterial culture media to immerse samples
- Survey the surface of samples with SEM

Rationale: Bacterial adhesion can result in development of peri-implant mucositis and peri-implantitis. Micro-organisms found in the oral cavity and their characteristics are widely studied, but the effects of these bacteria binding to the surface of an implant are not fully understood. Previous studies illustrate microbial adhesion to implant surfaces causing damage and leading to eventual implant failure [90]. It has also been reported that bacterial adhesion on implant surfaces can create an acidic electrochemical environment due to release of metabolic products such as lactic acid [90]. In order to investigate the effects of bacterial adhesion on titanium surfaces, the goal of this study is to develop an *in vitro* methodology that will allow for examination of an implant surface after exposure to bacteria. Samples immersed in bacterial culture will be compared

to control surfaces (not immersed/ not contaminated) to investigate for any changes using scanning electron microscopy (SEM) and optical microscopy (OM). Discoloration, bacterial metabolic depositions, pits and other areas of depth will be investigated as a result of the immersion procedure.

Hypothesis: Immersion of titanium surface in mixed bacterial strain culture will lead to biofilm growth on sample surfaces. Bacterial adhesion will create an acidic environment due to production of lactic acid, which will result in surface oxidation.

GOAL 2: To carry out detoxification of contaminated samples by immersion and rubbing procedures using chemicals typically employed in the clinical setting.

- Develop a protocol to carry out use of all chemicals and different detoxification methods
- Determine how to carry out experimentation without causing contamination of samples and lab environment
- Determine optimal time period for rubbing and immersion methods

Rationale: When an implant is affected by peri-implantitis, a clinician has two options for treatment: either remove the infected implant or proceed with other treatment methods. Peri-implantitis treatment methods employ chemical, mechanical and laser approaches to remove adhered biofilm. The most common method is debridement/detoxification of implant surfaces with

acidic chemicals to remove the biofilm. This is concerning because in order for re-osseointegration of bone to implant to occur post-treatment, it is important that the implant surface provides a fostering environment for bone-forming cells to grow on. If this is successful, the implant will have a greater chance of survival. Furthermore, it should also be mentioned that adhesion of bacteria can create an acidic environment leading to chemical attack and damage on the surface oxide layer. Hence, it is important to evaluate if detoxification agents can exacerbate the degrading effects already initiated by adhesion of bacteria on the implant surface. The aim in this stage of the project is to simulate the detoxification method using Q-tips dipped in chemicals (citric acid (40%), doxycycline (50:50), chlorhexidine (0.1%) and saline (0.9%) to manually scrub (rubbing method) off the biofilm formed on the sample surface from the experimental tasks described in Aim 1 (contamination with bacterial biofilm). Another method is to immerse (immersion method) the contaminated samples in the chemical for the same time period.

Hypothesis: Low pH of chemical agents used in detoxification procedures will create an acidic environment, which will cause oxidation, discoloration and pitting of titanium surfaces.

GOAL 3: To evaluate viability, differentiation and proliferation rates of bone-forming cells after detoxification.

- Develop a protocol for evaluating pre-osteoblast differentiation and proliferation, and cell staining on sample surfaces
- Carry out calculations to determine differentiation, and proliferation rates

Rationale: Bone-implant contact (BIC) plays a fundamental role in the long-term success and functionality of an implant [96]. Bone loss due to surgery or peri-implantitis must be replenished by re-osseointegration to achieve sufficient bone-to-implant contact. Extensive research has been done to study surface features and conditions that can affect integration with bone. But very few studies have investigated how growth of bone-forming cells on implant surfaces is affected by surface changes that may occur as a result of the combined effects of manual mechanical forces and chemicals used in the detoxification process. The rational for this experimentation is to investigate the same.

Hypothesis: Acidic chemical agents used as detoxification methods will lead to significant changes in the surface oxide layer decreasing cell proliferation and differentiation of pre-osteoblast cells on specimen surfaces.

CHAPTER 4

DETOXIFICATION OF TITANIUM IMPLANT SURFACES: EVALUATION OF SURFACE MORPHOLOGY AND PRE-OSTEOBLAST CELL COMPATIBILITY

4.1 ABSTRACT

Adhesion of bacterial biofilm on implant surfaces is considered as a primary cause of peri-implantitis. Mechanical debridement is commonly carried out to treat peri-implantitis-affected implants. The purpose of this study was to investigate the effect of bacterial adhesion and detoxification with acidic chemicals on titanium surfaces in addition to evaluating pre-osteoblast cell viability and proliferation post-detoxification to evaluate likelihood of re-osseointegration. The study consisted of 3 steps using polished titanium samples (n=36): (1) Contamination: polished titanium specimens were immersed in mixed bacterial strain containing *Aggregatibacter actinomycetemcomitans, Streptococcus mutans, Streptococcus sanguinis*, and *Streptococcus salivarius* for 5 days to allow formation of a biofilm on surfaces; (2) Detoxification: rubbing and immersion methods were employed to carry out detoxification using citric acid (30%), chlorhexidine (0.12%), doxycycline (50:50) and saline (0.9%) for 8 minutes; (3) Cell study: cultured pre-osteoblasts were placed on cpTi surface at a seeding density of $0.05x10^6$ cells/well and grown for 7 days. MTT and ALP assays were done after 7 days to examine proliferation and differentiation rates. Surface analysis of specimens was done using scanning electron microscope (SEM) and optical microscopy (OM) to verify signs of discoloration, corrosion, and pits. MTT

assay results for rubbing method revealed citric acid having the highest cell viability (86%) compared to saline (77%), while doxycycline and chlorhexidine resulted in 53% and 57% cell viability, respectively. ALP activity was highest for saline with rubbing treatment (0.00528 U/ml) and citric acid had the second highest ALP activity of 0.00524 U/ml. Optical microscopy images showed discoloration and minor pitting on sample surfaces that underwent rubbing method treated with citric acid and chlorhexidine. The synergistic activity of bacterial contamination and detoxification with acidic chemicals produced relatively low cell viability and proliferation rates. This suggests high toxicity of detoxification method on pre-osteoblast cells. The detoxification treatment produced noticeable discoloration and pitting attack on the titanium surface.

4.2 INTRODUCTION

Dental implants have altered the face of dentistry over the last 25 years. More than 500,000 implants are placed every year [2] with a reported success rate of approximately 90-95% [4], as mentioned in previous chapters. Success of a dental implant is primarily assessed by the condition of the implant's surface and its capability to biologically integrate with the surrounding soft and hard tissues [97]. But even with a high success rate, 5-10% of implants still fail, which result in economical and health burden to a large number of patients [4]. In general, dental implant failures are classified into early and late stage failures, with growth of bacteria being the ultimate factor in establishing which category of failure an implant falls into.

However, if peri-implantitis does occur, the clinician has the option to either remove the infected implant or perform debridement and detoxification of the implant surfaces to remove bacteria and its metabolites present on the surface in order to re-establish osseointegration [15]. There are many

different methods to treat implants affected by peri-implantitis including: chemical, mechanical and laser treatments [15]. Chemical treatment is employed for debridement of surfaces with biofilm; common chemicals used include citric acid, tetracycline, saline, chlorhexidine, hydrogen peroxide and doxycycline [27, 19, 28-30]. These chemicals are used along with mechanical means such as Er: YAG, CO_2 lasers, curettes, and powder blasting [15]. However, mechanical debridement with chemicals is most preferred and the most common treatment method for peri-implantitis.

The solutions used to facilitate debridement are effective at removing bacteria from the surface [93]. However, many of these solutions are low in pH and high in fluoride concentration, which are known to cause damage to the surface of titanium [89]. Though both prescribed oral mouthwash and detoxification treatments are effective for biofilm removal, they do not focus on the recovery of the de-osseointegrated interface between supporting bone and implant surface. This is significant because quality and condition of an implant depends on its surface properties such as osseointegration, corrosion resistance and biocompatibility [98].

Understanding the effects bacterial adhesion and peri-implantitis detoxification treatment method have on implant surfaces is crucial to drive innovations in implant design and to better inform clinicians performing such procedures in their practices. There is a lack of controlled *in vitro* studies that investigate the synergistic impact of bacterial adhesion and detoxification treatments on cellular growth. In this work, a new testing methodology was developed to investigate the surface performance of titanium when exposed to peri-implantitis inducing bacteria and commonly used chemicals for the detoxification method that simulate oral conditions and treatment: (1)

exposure to bacterial biofilm, (2) treatment with acidic chemicals along with mechanical debridement and, (3) exposure to host cells. The study's main goal was to investigate the impact of this sequence of events on the surface of titanium and speculate about the possibility of re-osseointegration post-detoxification treatment. The hypothesis was that the combined effect of mechanical abrasion with acidic chemicals would hinder growth of bone-forming cells. The experimental model developed also has the versatility to accommodate different dental implant materials as well as different peri-implantitis treatment methods. In summary, the results demonstrated that when titanium surface was mechanically rubbed with chemicals, there was higher cell viability compared to surfaces that were not subjected to mechanical abrasion.

4.3 MATERIALS AND METHODS

Commercially pure grade 2 titanium (cpTi) stock cylinders (McMaster-Carr, Elmhurst, IL, USA) was used in this study. Chemicals typically used by clinicians for debridement to treat peri-implantitis-infected implants were investigated. These chemicals included: citric acid monohydrate (Fisher Scientific, Hampton, NH) prepared at 30% concentration using deionized (DI) water, chlorhexidine gluconate (Chlorheximed GSK, Middlesex, UK) of 1%, saline (0.9%), and doxycycline (Actavis, Dublin, Ireland) prepared at a 50% with deionized water (DI) water.

In order to achieve the goals of the study, 4 main experimental steps were developed. A total of 33 commercially pure (cpTi) samples were used. The overall methodology is summarized in Figure 4.1.

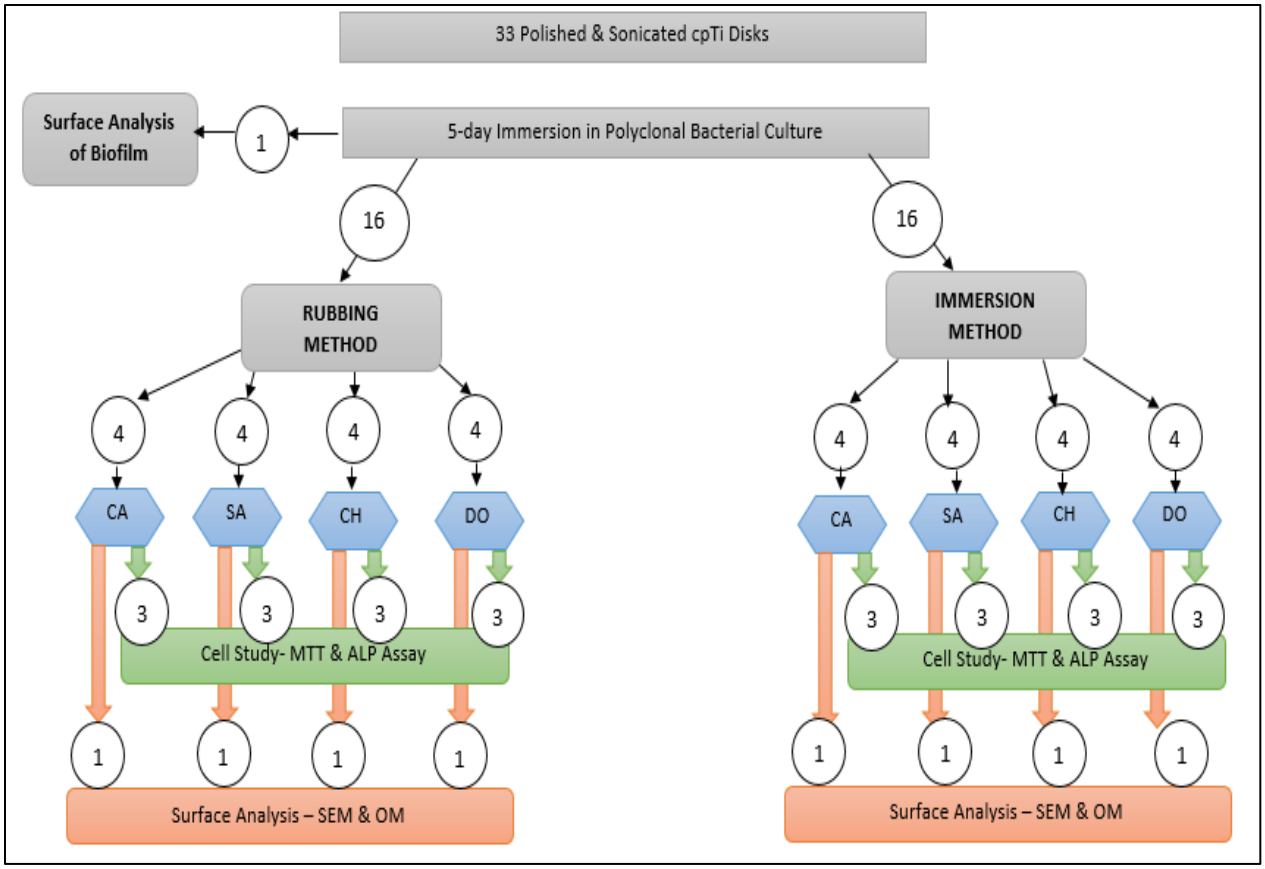

Figure 4.1 Scheme of the experimental procedure. Numbers in circles represent number of cpTi samples undergoing detoxification treatment method. CA-citric acid (40%), SA- saline (0.9%), CH-chlorhexidine (0.1), DO-doxycycline (50:50). (SEM: scanning electron microscopy) (OM: optical microscopy).

4.3.1 Preparation of Samples

Commercially pure grade 2 titanium (cpTi) stock cylinders were cut into 3-4cm diameter discs with 3mm thickness (McMaster-Carr) using a MEGA-M250 Manual Metallographic Abrasive Saw (Pace Technologies). The individual cpTi samples were then placed into re-usable plastic molds with a diameter of 1 inch as seen in Figure 4.2 (a). 5 ml of Ultrathin 2 Hardener-(ULTRA-3000H-08) and ULTRATHIN 2 Epoxy Resin in a 1:10 ratio was added into the mold. Samples were left to harden in the molds for 12 hours and then pulled out using screw driver and hammer,

as illustrated in Figure 4.2 (b). Next, manual polishing for each test specimen was done using a Penta 5000 Hand Grinder mounted with 240-grit until the surface of the sample was smooth and edges were levelled. Subsequently, the specimens were polished using abrasive silica paper (Pace Technologies) at 360, 600, 800 and 1200-grit for 4-8 minutes using the same hand grinder (Pace Technologies). Sequentially, the samples were fine-polished with alumina and diamond micro polish on velvet cloth (Nano 1000T, Pace Technologies). Next, samples were carefully removed from the epoxy mold using a Rock Rascal-RR JM, 6-inch saw (Model JM-Johnson Brother). Then, specimens were immersed face up in a 100 ml beaker filled with 10 ml acetone and placed in sonicator (Branson 3800) for 15 minutes. Subsequently, acetone was discarded and de-ionized water added for an additional 15 minutes of sonication. The same procedure was repeated with ethanol. After sonication was complete, samples were placed in a clean beaker, covered with foil and placed in oven (Symphony VWR) at 60°C overnight.

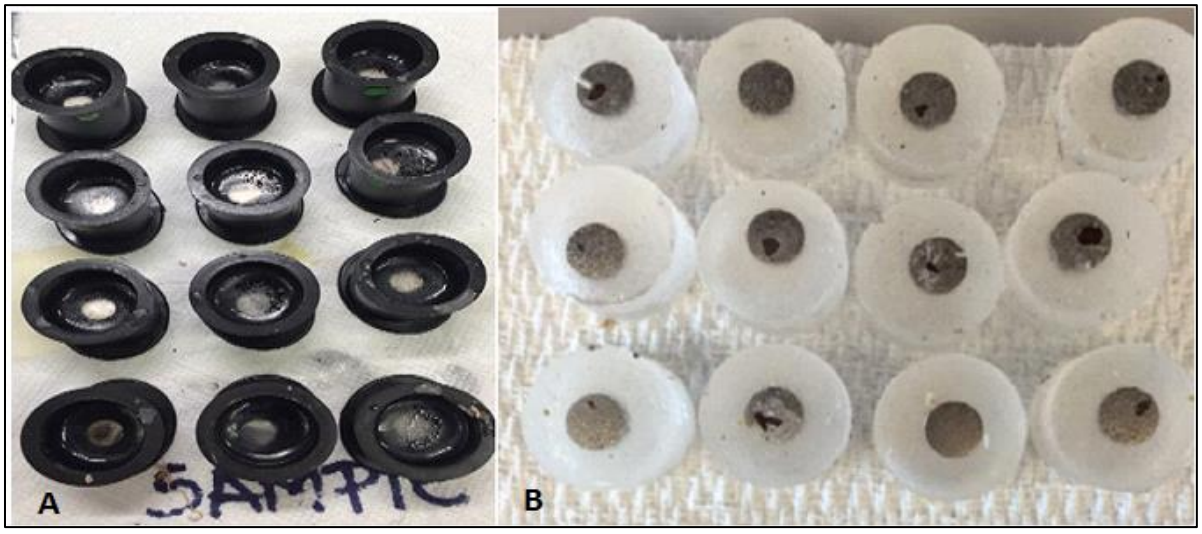

Figure 4.2 Individual cpTi samples placed in molds with hardener and epoxy resin (A) and then removed from the mold after 12 hours (B).

4.3.2 Contamination of Titanium Surface

The combination of early colonizing *Streptococcus mutans, Streptococcus sanguinis, and Streptococcus salivarius* and late colonizing *Aggregatibacter Actinomycetemcomitans* (Aa) was selected to develop a clinically relevant peri-implantitis-inducing environment for the study.

4.3.2.1. Bacterial Culture Preparation and Sample Contamination

Streptococcus mutans (UA 159), Streptococcus sanguinis (10556), Streptococcus salivarius (13419) and late colonizing A*ggregatibacter Actinomycetemcomitans (VT 1169)* were cultured in brain heart infusion (BHI) agar plates (BD, Franklin Lakes) and incubated at 37°C in 5% CO_2 microaerophilic condition (BD GasPak) for 48 hours until colonies were formed. Next, individual colonies from each strain of bacteria were inoculated into 5 ml BHI broth medium in a 24 well plate to create a mixed culture. Each of the 33 cpTi samples were immersed in individual wells containing the mixed culture as shown in Figure 4.3. The plate was then incubated for 5 days at 37°C in 5% CO_2 microaerophilic condition (BD GasPak) to develop a biofilm. Turbidity of the BHI broth medium was checked every 48 hours. This time period was chosen to perform a short-term test for bacterial adhesion and biofilm growth. After 5 days, the specimens were removed from each well, and individually wrapped in aluminum foil and autoclaved.

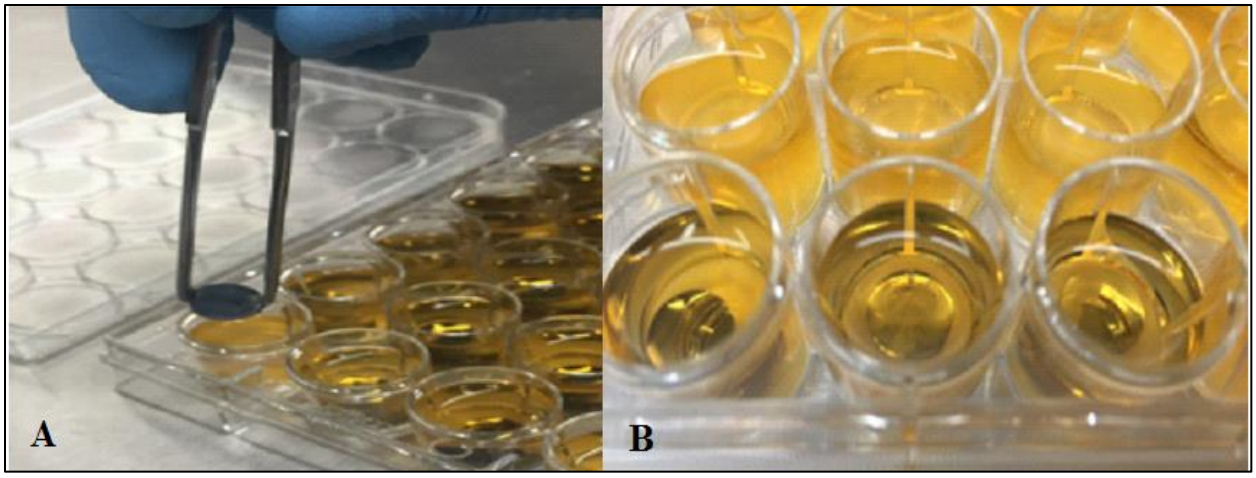

Figure 4.3 Process of immersion of cpTi samples into each well of a 24-welled plate containing bacterial strain (A); immersed samples (B).

4.3.3 Detoxification of Titanium Surface

4.3.3.1. Selection and Preparation of Chemicals

Citric acid (30%) (30g in 100 ml of deionized water) and chlorhexidine gluconate oral rinse solution (0.12%) were chosen for the study because they are commonly used chemicals for debridement and oral hygiene. Citric acid has an acidic pH of 1.74 while chlorhexidine has a basic pH of 7.2; this range would help to better understand the effect of acidic and basic chemicals on the surface. An animal study done by Takasaki *et al.* showed saline irrigation resulted in an increase in re-osseointegration [19, 99]. Therefore, saline was included in this study to compare resulting cell viability with the effect of other chemicals. Lastly, doxycycline (50:50; deionized water: doxycycline powder) was chosen because it is a commonly prescribed tetracycline antibiotic by orthodontists for periodontal diseases.

4.3.3.2. Rubbing and Immersion Method Treatment

The two methods employed for detoxification process in this study were: rubbing and immersion. Rubbing method involved soaking a cotton swab in the chemical solution and manually rubbing the sample surface in a circular motion as seen in Figure 4.4 (B); mimicking mechanical abrasion done by clinicians during detoxification of implant surfaces. With the immersion method, cpTi specimens were immersed in the selected chemical to mimic irrigation of dental implant when exposed to chemical solutions as seen in Figure 4.4 (A). Each technique was carried out for 8 minutes. Detoxification treatments not only differ from clinician to clinician, but also depend on the progression of peri-implantitis, which differs from patient to patient. Some clinicians will carry out debridement for 1 minute, and then take a break and then do another set of 1-minute, with a net debridement time from anywhere between 1 minute to 10 minutes. Because of the variation in treatment time, this study included a debridement time of 8 minutes to represent a worst case scenario; wherein, the implant would be rubbed or immersed with the chemical for a net period of 8 minutes to remove heavy build-up of plaque and bacteria. For each selected chemical, a group of 4 cpTi samples underwent immersion treatment and another group of 4 cpTi samples received the rubbing treatment. Of the 4 in each method, 1 was used for surface analysis using optical microscopy (OM) (Keyence VHX–2000) and scanning electron microscopy using a range of 5-20 kV (SEM, JEOL JSM-6010). The rest of the 3 samples in the group of 4 were used to carry out cell studies.

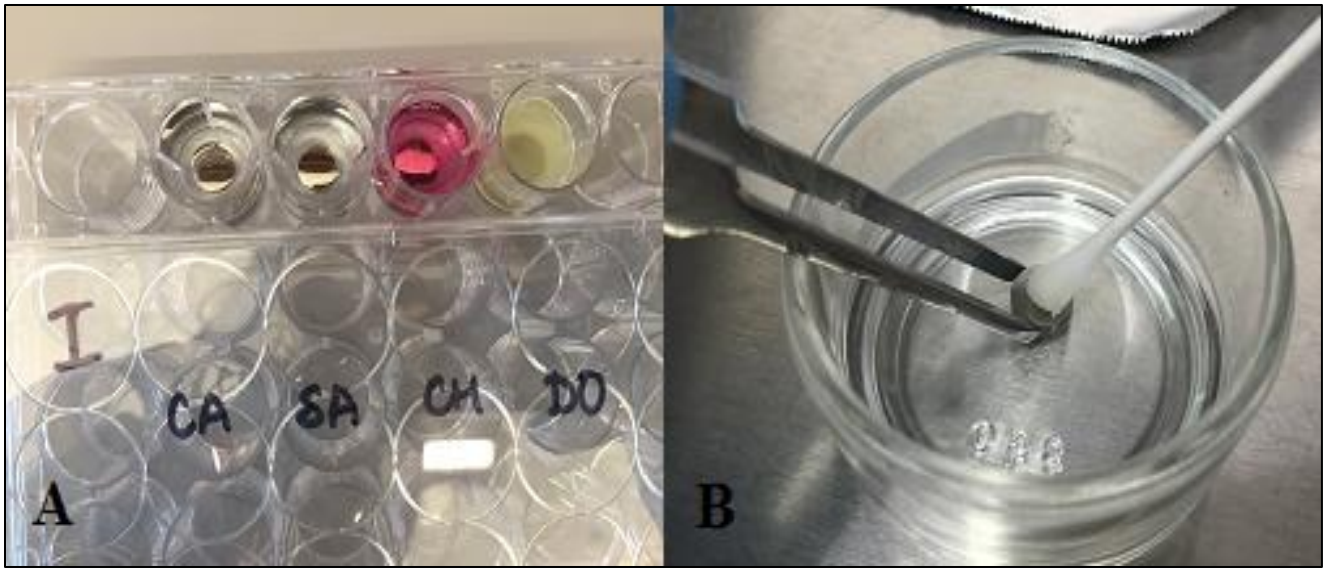

Figure 4.4 (A) Immersion method- cpTi samples immersed in CA-citric acid (30%), SA-saline (0.9%), CH-chlorhexidine (0.1%), and DO-doxycycline (50:50). (B) Rubbing method- a cpTi sample undergoing rubbing treatment with a Q-tip dipped in chemical.

4.3.4 Evaluation of Cell Compatibility on Detoxified Surfaces

4.3.4.1. Pre-osteoblast (MC3T3-E1) cell growth

Cultured pre-osteoblasts (MC3T3-E1) (American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium Alpha Modification (1X) media (American Type Culture Collection) supplemented with 10% Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences) in T-75 flasks. All cell culture studies were performed *in vitro* using 24-well culture plates. Once the samples underwent detoxification, each sample was placed individually in a well of a labelled 24-well plate. Pre-osteoblast cells (MC3T3-E1) were placed on each cpTi surface at a seeding density of 0.05 x 10^6 cells/well along with 1 ml of Dulbecco's Modified Eagle's Medium Alpha Modification (1X) media (American Type Culture Collection) supplemented with 10% Fetal

Bovine Serum (FBS) (GE Healthcare Life Sciences). Cells were incubated at 37°C for 7 days; media in each well was changed every two days.

4.3.4.2. MTT Assay

After 7 days, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide assay was performed to evaluate cell viability for each chemical and detoxification method performed. Non-treated samples were used as control for comparison of cell viability observed with treated samples. First, media from each well in the 24-well plate was aspirated, cells were washed with PBS and then typsinized to detach the cells from the bottom of the wells and cpTi surface. Unattached cells from each well were transferred into separate 50 ml centrifuge tubes with 1 ml media and centrifuged at 1200 rpm for 5 minutes after which the supernatant was removed. Cells were re-suspended in 250 µl of media. 100 µl of this cell solution, from each centrifuge tube, was added to separate wells in 96-well plate to carry out MTT and 100 µl of Dulbecco's Modified Eagle's Medium Alpha Modification (1X) media was added into 3 separate wells as blank control. To each well, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent was added in the dark and the 96-welled plate was incubated at 37°C for 4 hours. After 4 hours, 100 µl of detergent reagent was added and the plate was placed in dark environment overnight. Optical density was measured using an automatic plate reader (Synergy Mix, Biotek) after 12 hours at 570 nm. Intensity of the blue formazan produced by the cells results in distinct optical density values. These values were used to calculate percentage cell viability after the blank optical density was subtracted.

4.3.4.3. ALP Assay

Alkaline phosphatase (ALP) assay is a colorimetric assay that measures the amount of ALP created by cells. High ALP activity signifies the differentiation of these cells from pre-osteoblasts to osteoblasts. After proliferation, pre-osteoblasts start differentiating into osteoblasts, at this stage there is a high expression of alkaline phosphatase (ALP). ALP assay helps to quantify the extent of differentiation found among the cells. In addition to the ALP assay, ALP staining was done to visually examine whether or not cells attached to cpTi surface.

From the 250 µl cell suspension obtained after centrifuging in section 4.3.4.2, 50 µl was added to a separate 96-well plate for ALP Assay (Abcam), in addition to 50 µl of the Dulbecco's Modified Eagle's Medium Alpha Modification (1X) media in 3 other wells as blank. Next, 30 µl of assay buffer and 50 µl of pNPP solution was added to each well. At this point, 20 µl of stop solution was transferred to the blank wells only. Then the plate was incubated for 1 hour in dark environment at room temperature. Afterwards, 20 µl of stop solution was added to the rest of the wells and optical density was read at 405 nm. With these readings, ALP activity was calculated with the calibrated curve.

4.3.4.4. ALP Cell Staining

A 7-day ALP staining procedure was carried out on treated samples to observe pre-osteoblasts growth on cpTi surfaces. Samples treated with citric acid (30%), saline (0.9%), chlorhexidine (0.1%), and doxycycline (50:50) were placed into individually in a 24-well plate. Pre-osteoblast cells were placed on cpTi surfaces at a seeding density of $0.05x10^6$ cells/well and grown for 7 days, changing media every 2 days. After 7 days, media was aspirated and each well was rinsed

with PBS and then aspirated. 2 ml of neutral buffered formalin (10%) was added into each well to cover the monolayer of cells. After 60 seconds, the formalin was aspirated and cells were washed with washing buffer (0.05% Tween 20 to Dulbecco's PBS, w/o Ca2+/Mg2+) and then aspirated again. 2 ml of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (My BioSource) substrate was added to immerse the monolayer of cells and then the plate was incubated in the dark for 20 minutes; every 5 minutes the plate was checked for the purple color stain. Once the color was seen, the solution was aspirated, then washed with washing buffer and this was aspirated also. Next, 2 ml of PBS was added into each well and the stained cells were analyzed using optical microscopy (OM) (Keyence VHX – 2000).

4.3.5 Surface Analysis of cpTi Samples

Surface analysis of cpTi surface was conducted at each step of the procedure. As illustrated in Figure 4.1, a total of 9 cpTi specimens underwent surface analysis with optical microscopy and scanning electron microscopy. Optical Microscopy images were taken using high dynamic range (HDR) setting on the microscopy using both low and high magnifications (up to 1000X). Images were taken at multiple points on the cpTi surface to visualize any morphological changes compared to the control (non-treated samples). Scanning electron microscopy images were taken using a range of 5-20kV.

4.3.6 Statistical Analysis

Statistical Analysis was performed using one-way analysis of variance (ANOVA) with Origin Pro 8 Software. Statistical significance was observed when the p-value was 0.05 or less (95% confidence level).

4.4 RESULTS

4.4.1 Surface Analysis of cpTi Surface

As illustrated in Figure 4.1, 33 cpTi specimens were immersed in mixed bacterial culture for 5 days. After 5 days, 1 sample was used to analyze surface changes after bacterial adhesion. The remaining 32 samples were separated into 2 different groups, each group containing 16 cpTi specimens. 1 group of 16 samples underwent rubbing treatment, and the other group underwent immersion with chemicals. Each group of 16 samples were further divided into 4 groups of 4 samples each that underwent treatment with a chemical: citric acid, saline, chlorhexidine and doxycycline. After detoxification treatment of these samples, 3 of the samples was used to execute cell studies while the remaining 1 was used to perform surface analysis to visualize any morphological changes to the surface post-treatment. The following section shows images taken with scanning electron microscopy (SEM) and optical microscopy (OM) to corroborate findings. Each image is for 1 of 2 cpTi specimen that underwent a particular treatment method.

4.4.1.1 Surface Evaluation of Contaminated Samples

The goal of this experimental procedure was to compare the morphology of contaminated cpTi surfaces with uncontaminated cpTi (control). CpTi samples were contaminated using mixed

bacterial culture containing *S. mutans, S. salivarius, S. sanguinis, Aggregatibacter actinomycetemcomitans* for a period of 5 days to allow for biofilm growth on cpTi surfaces. Figure 4.5 shows cpTi samples that were immersed in the mixed bacterial culture grown in BHI medium. On day 1 of immersion, BHI medium was observed to have a clear yellow coloration as seen in Figure 4.1 (A). Figure 4.1 (B) shows cpTi samples after 5 days of immersion. Here, a thin white bacterial film was visible on sample surfaces.

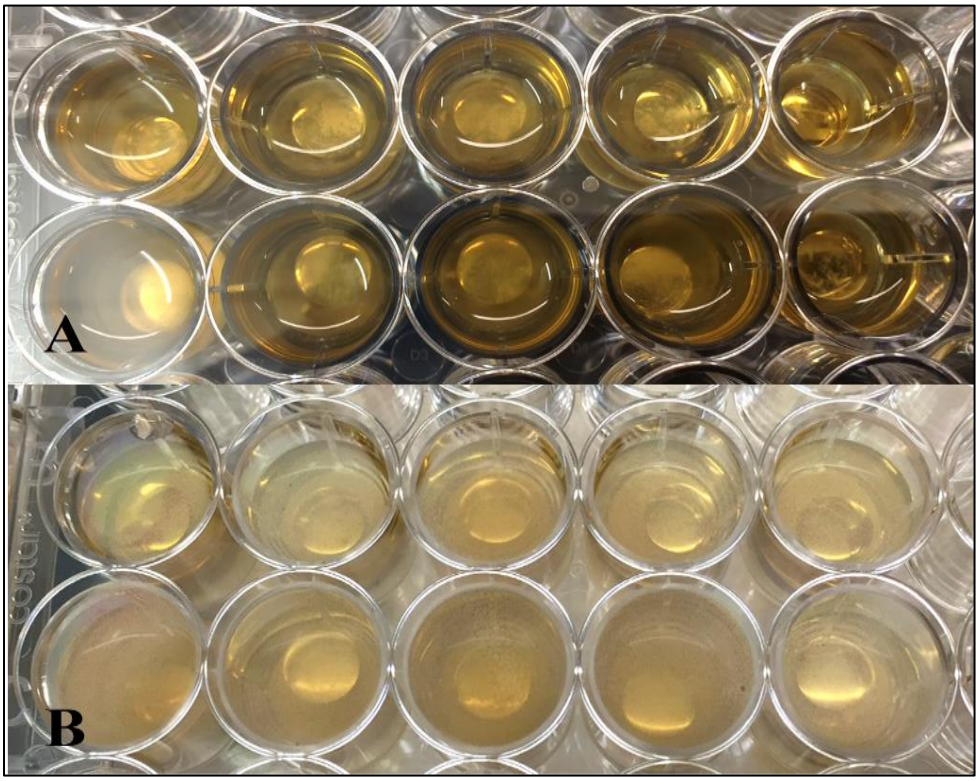

Figure 4.5 Digital image of immersed cpTi samples in BHI medium with *S. mutans*, *S. salivarius*, *S. sanguinis*, Aa bacteria. (A) Day 1 of immersion; (B) Day 5 of immersion.

Similarly, a white film could be observed on the surface of titanium samples after they were removed from the immersion medium as shown in Figure 4.6. Turbidity and color of the BHI medium were observed for any changes during the immersion period to ensure no contamination was present. Contamination was detected if the media changed from a clear yellow to turbid yellow

color. But, because the medium was not disturbed during the 5 days of incubation, contamination was not an expected issue.

Figure 4.6 A contaminated cpTi sample with a visible white biofilm on the surface.

Figure 4.7 is an SEM image of 1 contaminated cpTi sample compared to control cpTi (A). Figure 4.7 (B) shows the SEM image of clusters of bacteria adhered to the specimen surface, which was seen like a film covering the surface. Figure 4.7 (C) shows the SEM image of the same sample with biofilm removed by sonication. A pit-like feature was observed and is indicated by the yellow arrow in the figure. Correspondingly, Figure 4.8 is the OM image of the same sample that underwent SEM imaging. In Figure 4.8, severe discoloration (yellow and blue) was observed around bacteria clusters on the surface (Figure 4.8 B) and more so after the biofilm was removed (Figure 4.8 C) as indicated by the yellow arrows.

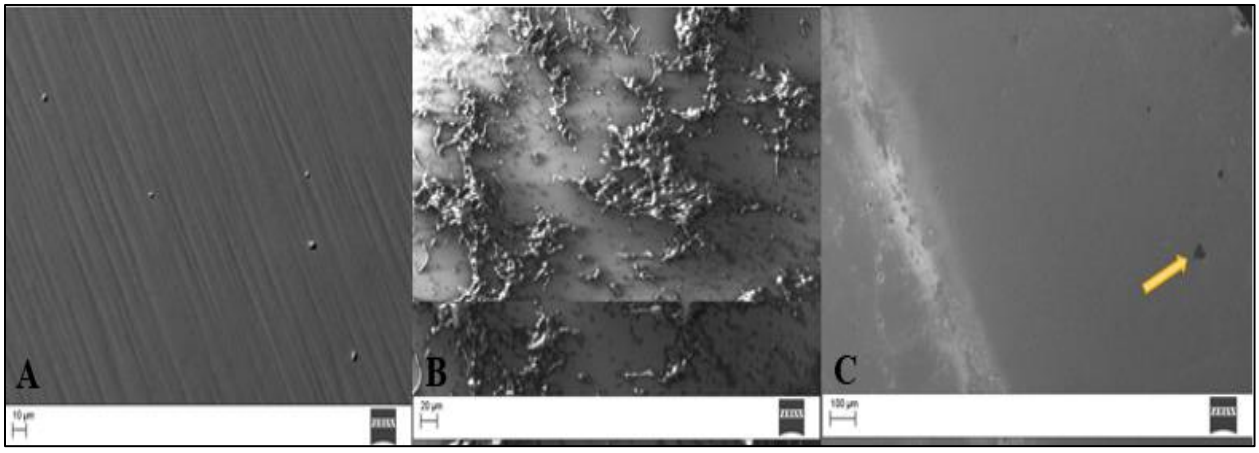

Figure 4.7 SEM image of uncontaminated sample (A), contaminated with biofilm on surface (B), contaminated and biofilm removed (C).

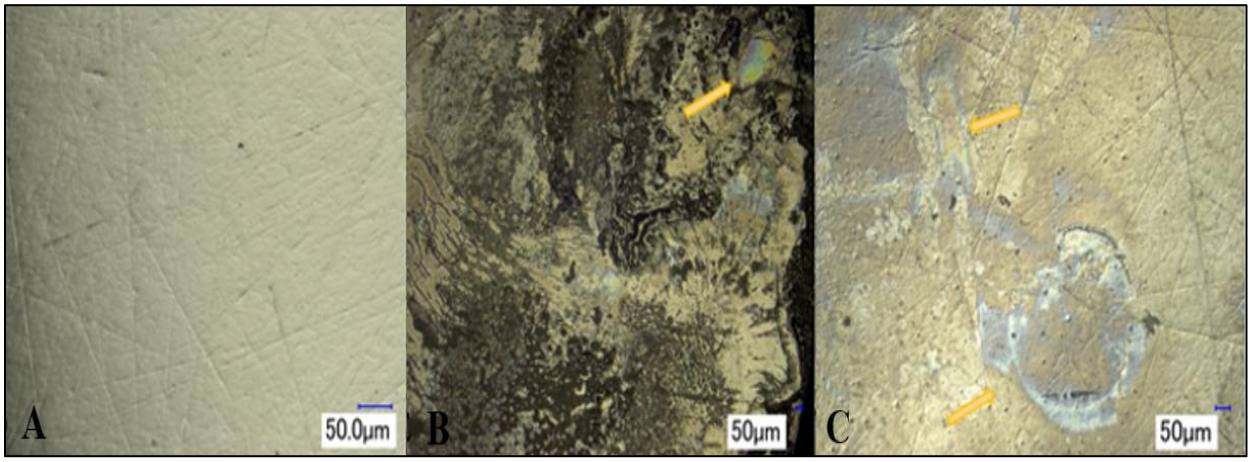

Figure 4.8 OM image of uncontaminated sample (A), contaminated with biofilm intact (B), contaminated with biofilm removed (C).

4.4.1.2 Surface Evaluation of Treated Surface

Chemicals included in this study had a pH range from very acidic to neutral. Table 4.1 lists the pH of each chemical measured before treatment. Citric acid being the most acidic followed by doxycycline, chlorhexidine and saline being the most neutral chemical.

Table 4.1 pH of each chemical used to carry out debridement via rubbing and immersion.

Chemical	**pH**
Citric Acid (30%)	1.74
Doxycycline (50:50)	2.74
Chlorhexidine (0.1%)	7.38
Saline (0.9%)	7.44

4.4.1.2.1 Surface Evaluation of Samples Treated by Rubbing Method

Figure 4.9 is the surface analysis images of 4 samples that endured rubbing with a chemical. SEM and OM were performed for each sample to visualize and compare changes inflicted on the surface.

Citric acid, being the most acidic among the other chemicals in the group, inflicted the most significant damage to the surface of titanium compared to the other chemicals investigated. Severe discoloration and pitting attack were observed with this treatment and can be seen in the SEM and OM images illustrated in Figures 4.9 (B) and (G) as indicated by the yellow arrows.

Samples rubbed with doxycycline showed mostly minor pitting and no discoloration (Figure 4.9 (I)) indicated by the yellow arrow, but was restricted to a particular area on the surface. A lot of residue from the doxycycline chemical was seen on the surface as dark agglomerations in the SEM image (Figure 4.9 D).

Rubbing with chlorhexidine generated discoloration on the specimens as shown in the SEM image (Figure 4.9 E) and in the OM image (Figure 4.9 J). Samples treated by saline with rubbing (Figure

4.9 (C) and (H)) showed no discoloration or pitting, and were observed to exhibit similar surface features as control specimens (Figure 4.9 (A) and (F).

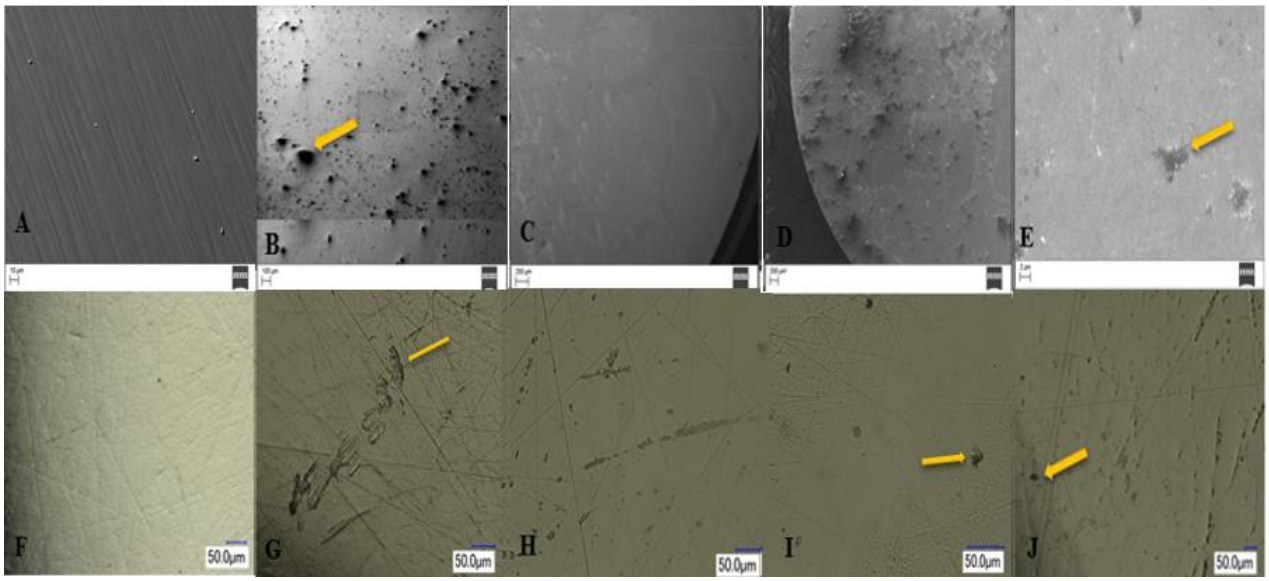

Figure 4.9 SEM (A-E) and OM (F-J) images obtained from 4 samples treated by rubbing method with a particular chemical. (A) SEM image of control specimen; (B) SEM image of citric acid treated; (C) SEM image of saline treated; (D) SEM image of doxycycline treated; (E) SEM image of chlorhexidine treated; (F) OM image of control sample; (G) OM image of citric acid treated; (H) OM image of saline treated; (I) OM image of doxycycline treated; (J) OM image of chlorhexidine treated.

4.4.1.2.2 Surface Evaluation of Samples Treated by Immersion Method

Figure 4.10 is the surface analysis images of 4 samples that were subjected to immersion with a chemical. SEM and OM were performed for each sample to visualize and compare changes to the surface.

Immersion in citric acid showed discoloration (indicated by yellow arrows) within cracks present on the surface, as illustrated in the OM image (Figure 4.10 G). As observed for samples subjected to rubbing, immersion in saline inflicted no negative impact on the surface of titanium specimens

(Figure 4.10 (C) and (H)) compared to the control specimen (Figure 4.10 (A) and (F)). Similar to the rubbing method, immersion in doxycycline, resulted in a significant amount of residue left on the sample surface as can be observed by the SEM (Figure 4.10 (D)) and OM (Figure 4.10 (I)) images. No significant morphological changes were observed for this specimen. Lastly, immersion in chlorhexidine created minor discoloration (blue and purple as shown by yellow arrows) illustrated in Figure 4.10 (J and E).

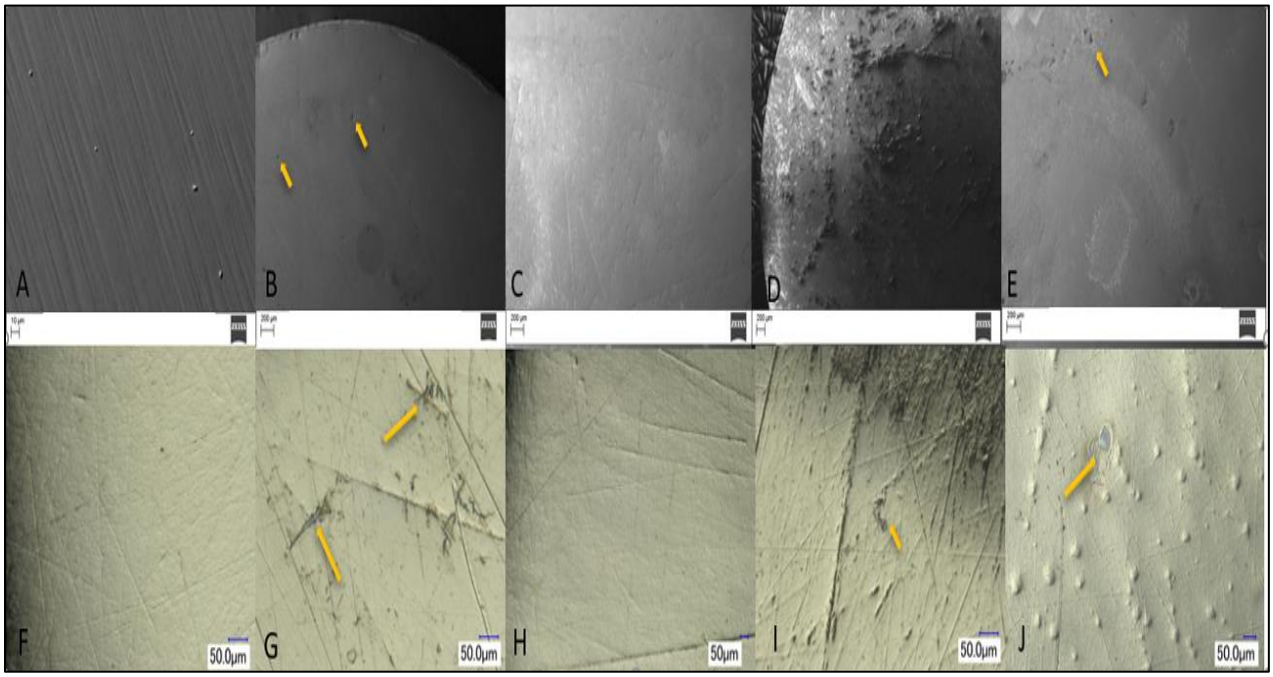

Figure 4.10 SEM and OM images obtained for samples treated by immersion method. (A) SEM image of control sample; (B) SEM image of citric acid treated; (C) SEM image of saline treated; (D) SEM image of doxycycline treated; (E) SEM image of chlorhexidine treated; (F) OM image of control specimen; (G) OM image of citric acid treated; (H) OM image of saline treated; (I) OM image of doxycycline treated; (J) OM image of chlorhexidine treated.

4.4.2 Quantification of Cell Compatibility on cpTi Surface After Detoxification

After treating the samples via rubbing and immersion methods using the various chemicals mentioned, samples were placed into 24-welled plates and pre-osteoblasts were seeded onto cpTi surfaces with media for 7 days, changing media every 2 days. After 7 days, 3[4,5-dimethylthiazol-2-y1]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Alkaline Phosphatase Assay (ALP) were carried out to assess cytocompatibility of the surfaces post-treatment.

Cytocompatibility of pre-osteoblasts to treated specimens was assessed using the ISO 10993-5:2009 standard. This standard provides a testing method to asses *in vitro* cytotoxicity of medical device surfaces and/or extracts of a device to cells in direct or indirect contact with these surfaces. The following are the levels of cell cytotoxicity set by the standard:

Table 4.2 ISO 10993-5:2009 standard for cell cytotoxicity of cells in contact with medical devices or extracts of the device.

Cell Viability (%)	Cytotoxicity Level
>80	Non-cytotoxic
80-60	Weak cytotoxicity
60-40	Moderate cytotoxicity
<40	Strong cytotoxicity

4.4.2.1 Cell Viability of Pre-Osteoblasts After Detoxification

Host cell response to treatment can be observed in Figure 4.11. Cell viability of pre-osteoblasts (MC3T3-E1) on sample surfaces were compared to the cell viability on non-treated samples

(control). The impact of rubbing (mechanical abrasion) against no mechanical abrasion (immersion) with each chemical can be compared in Figure 4.11.

Overall, the cell viability of pre-osteoblasts on samples that experienced abrasion (rubbing), was lower than on samples that did not experience abrasion (immersion) with the exception of citric acid. In addition, when comparing cell viability of cells on treated samples to cell viability on non-treated samples, viability was lower on treated specimens by rubbing with each chemical. Whereas for the immersion method, cell viability for saline and doxycycline exceeded that of the control, unlike citric acid and chlorhexidine which resulted in lower viability than the control.

There was a significant difference in cell viability between rubbing and immersion methods when treated with doxycycline ($p<0.05$), citric acid ($p<0.05$), and between saline-immersion and citric acid-rubbing ($p<0.05$). Although, saline immersion resulted in much higher cell viability compared to saline-rubbing, there was no statistical difference between these treatment methods ($p>0.05$). For chlorhexidine, evidence of significant difference between rubbing and immersion was not observed ($p>0.05$), although, rubbing with chlorhexidine induced lower cell viability than immersion with chlorhexidine.

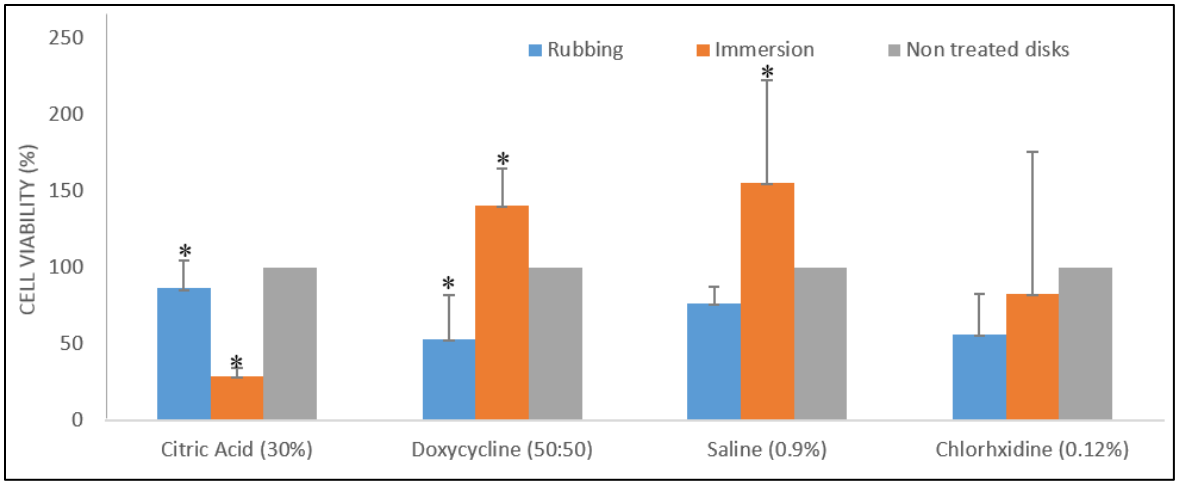

Figure 4.11 Cell viability of pre-osteoblasts on samples treated via rubbing and immersion, compared to non-treated control samples. *Reduction was statistically significant ($p<0.05$) (n=3).

4.4.2.2 Cell Differentiation of Pre-Osteoblasts After Detoxification

We further quantified osteogenic differentiation by measuring alkaline phosphatase (ALP) activity. This enzyme is an osteogenic differentiating marker that quantifies the rate of pre-osteoblast differentiation into osteoblasts with the help of p-nitrophenyl phosphate, which gets hydrolyzed by ALP into a yellow color substance. The optical density of this yellow color was measured at 405 nm and the rate of this reaction is directly proportional to ALP activity. Figure 4.12 demonstrates ALP activity of pre-osteoblasts on cpTi specimen post-treatment.

On average, higher ALP activity was seen on cpTi specimens that experienced mechanical abrasion (rubbing) than with samples that were immersed. No statistical difference ($p>0.05$) was found for ALP activity between rubbing and immersion methods, nor was there any statistical difference ($p>0.05$) between chemicals used for the two treatment methods.

Also, in general, ALP activity of samples immersed in all 4 chemicals was lower than the ALP activity on non-treated specimens (control). For samples rubbed with doxycycline and

chlorhexidine, the ALP activity was lower than the control, whereas samples rubbed with citric acid and saline exhibited ALP activity greater than that on non-treated samples.

From Figure 4.12 comparing between rubbing and immersion, doxycycline did not differ ($p>0.05$), while saline and chlorhexidine only slightly differed ($p>0.05$), with rubbing method resulting in higher ALP activity overall. Citric acid resulted in greater difference in ALP activity when samples were immersed compared to rubbing with the chemical, although no significant differences were found ($p>0.05$).

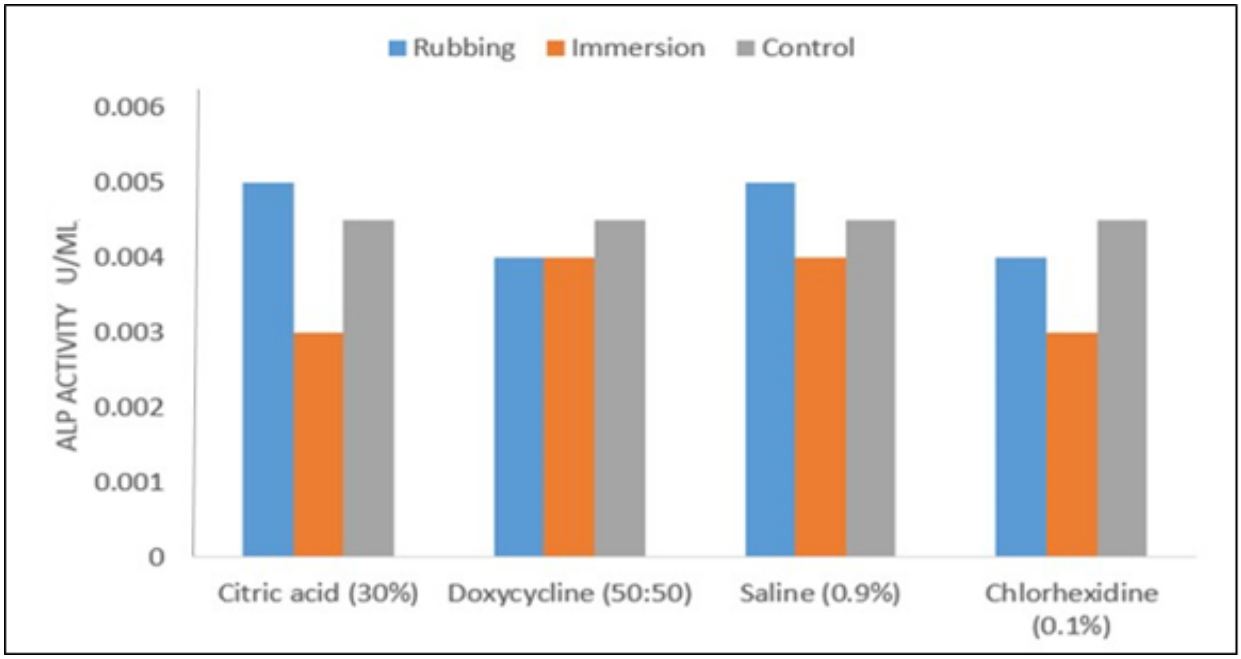

Figure 4.12 ALP activity (U/ml) of differentiated pre-osteoblasts. *Reduction was statistically significant ($p < 0.05$) ($n = 3$).

Adhesion of differentiated pre-osteoblasts on control samples and treated specimens was visualized using optical microscopy. This part of the study was done to observe how well differentiated pre-osteoblasts adhered to cpTi surfaces. There were a total of 8 specimens used for

this portion of the study, 4 samples subjected to rubbing with chemicals mentioned above, and the other 4 samples subjected to immersion in the chemicals.

All test specimen surfaces were stained to detect ALP enzyme and the differentiated cells were identified by the purple color of the stain as seen in Figure 4.13.

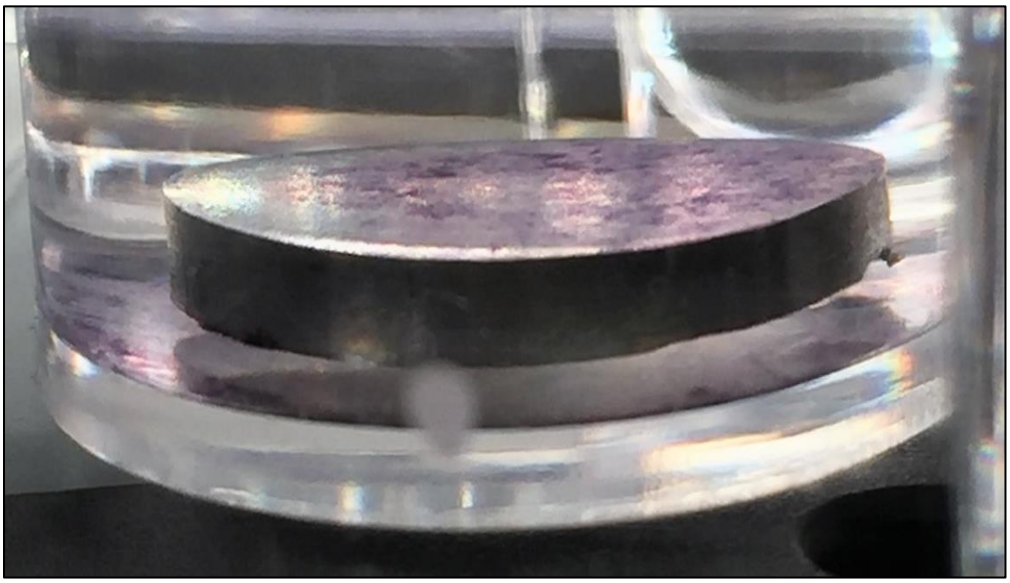

Figure 4.13 A cpTi sample surface with stained monolayer of differentiated pre-osteoblasts.

Figure 4.14 (A) shows the well with only media and cells, whereas Figure 4.14 (F) shows the non-treated sample surface with a monolayer of cells present. Samples treated via rubbing method seemed to have a lot more differentiated cells attached to the surface compared to immersion-treated samples. Even though citric acid was the most acidic, it was observed that both rubbing (Figure 4.14 (E)) and immersion (Figure 4.14 (J)) methods resulted in high coverage of differentiated cells. The same observation was obtained for specimens treated with saline (Figure 4.14 (D) and (I)). There was a significant amount of differentiated cells adhered to the surface of specimens treated by rubbing method using chlorhexidine (Figure 4.14 (C)).

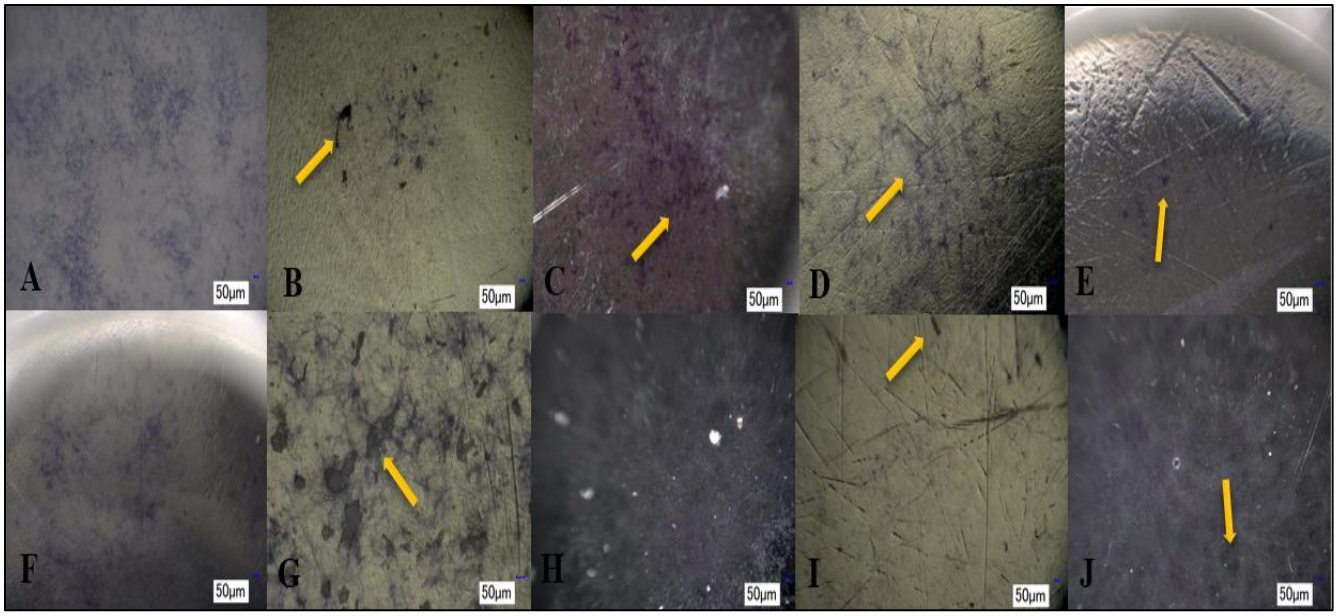

Figure 4.14 OM images obtained from ALP Staining (R-rubbing, I-immersion): (A) Media + cells, (B) R-doxycycline, (C) R-chlorhexidine, (D) R-saline, (E) R-citric acid, (F) Non treated sample, (G) I-doxycycline, (H) I-chlorhexidine, (I) I-saline, (J) I- citric acid.

4.5 DISCUSSION

The main goal of this study was to develop a short-term *in vitro* testing procedure to investigate the effects of bacterial adhesion and mechanical debridement on the morphology of cpTi surface. Subsequently, to assess growth of pre-osteoblast cells on titanium surface after the synergistic effects of bacterial adhesion and detoxification. The study was designed to simulate the human oral environment and detoxification treatment methods typically used by clinicians. This was done by contaminating titanium surfaces with peri-implantitis inducing bacterial strains and by implementing a mechanical debridement treatment method involving the use of a series of chemicals. There are numerous clinical studies that have examined the impact acidic chemicals

have on titanium surfaces [27, 29, 89], but only a few have looked at the synergistic activity of bacterial adhesion and mechanical debridement on growth of pre-osteoblasts.

With the first aim of study, it was hypothesized that immersion of titanium samples in mixed bacterial strain along with media would lead to growth of a biofilm on sample surfaces. Subsequently, it was hypothesized that this bacterial adhesion on cpTi would create an acidic environment due to production of lactic acid, and a crevice-like environment, which would result in oxidation of the surface.

The incorporation of early colonizing and late colonizing bacteria (*S. mutans, S. sanguinis, S. salivarius* and *Aggregatibacter actinomycetemcomitans)* was chosen to ensure clinical relevance to the oral cavity of a peri-implantitis infected patient. *S. mutans* is a gram-positive bacterium, which is one of the primary colonizers of biofilm on tooth surfaces and is the most abundant bacteria found in peri-implant tissue compared to periodontal microflora [100]. *S. mutans* along with the other bacteria are known to create acidic environments in the mouth as a result of metabolizing carbohydrates from food intake due to the release of organic acids [101]. Metabolites such as lactic acids are produced by bacteria, which can contribute to the reduction of pH. Even though titanium has a naturally forming oxide layer, which protects against corrosion, if this layer gets disturbed or covered by bacterial adhesion, continuous metal dissolution and corrosion may occur, which can be identified by surface features such as discoloration, pitting attack, and delamination

It has been shown that the presence of bacteria on implant surfaces can reduce the pH and may contribute to oxidation of the implant surface [102, 103, 104]. In previous studies conducted by

Sridhar *et al.* [93] and Rodrigues *et al.* [90] two possible mechanisms of corrosion involving bacteria have been proposed: (1) after adhesion and during glycolysis, early colonizing planktonic bacteria release lactic acid which decreases the pH of the oral environment. When titanium experiences low pH, the oxidation state of its surface changes leading to metal ion dissolution; (2) once a biofilm is formed on a surface, a crevice environment is created with restricted aeration and fluid exchange. This creates localized oxygen-depleted zones, wherein within these crevices pH is further decreased subsequently resulting in accelerated metal dissolution.

Results from the first step of this study revealed that bacterial adhesion does indeed change the morphology of titanium surface. Optical microscopy and scanning electron microscopy showed severe discoloration and pitting attack along the bacterial adhesion agglomerates found on sample surfaces. Once the biofilm was removed by sonication, discoloration was more prominently observed throughout specimen surfaces. This can be corroborated with previously mentioned mechanisms of corrosion triggered by bacteria. The non-uniform biofilm layer created oxygen depleted zones resulting in crevice-corrosion, which was observed by the yellow and blue discoloration of the surface found around bacterial agglomerates. Moreover, secreted lactic acid from adhered biofilm contributed for the observed effect.

In the second aim of the study, detoxification of contaminated samples was carried out. Currently, there is an ongoing debate in literature about the effectiveness of this method in the treatment of peri-implantitis infected implants. This is because there is a lack of agreement to which chemical agent and technique is the most efficient to treat this condition. Furthermore, this is aggravated by the fact that current studies greatly differ in implant type, concentration of chemical and technique

used to detoxify implants [27, 29, 89]. But the majority of these clinical or *in vitro* studies show some evidence of change in morphology of titanium surfaces after detoxification with acidic chemicals.

In the present study, discoloration and pitting were mainly observed when surfaces were treated with citric acid and doxycycline, which were the two most acidic chemicals. The morphological changes were more prominently observed for samples that were rubbed with these two chemicals than immersed in them. Chlorhexidine and saline, two of the neutral chemicals evaluated, inflicted little to no effect on specimen morphology, with both rubbing and immersion methods.

Similarly, to this study, the impact of mechanical motion on titanium surfaces has been evaluated and established that mechanical factors can play a significant role in the degradation of titanium surfaces and more-so the synergistic action of electrochemical and mechanical factors [93]. A previous study conducted by Wheelis *et al.* [89], evaluated the effects of 3 chemicals, peroxyacetic acid (35% in acetic acid, pH ~0), citric acid (40% in D.I. water pH = ~1), and 0.12% sodium fluoride (in D.I. Water pH ~8), on cpTi and Ti-6Al-4V alloy samples. In the study, the same treatment methods were employed (rubbing and immersion) for 8 minutes. Optical microscopy and Atomic Force Microscopy (AFM) revealed that acidic chemicals (pH<3) inflicted mild corrosion on surfaces immersed in the chemical. Whereas specimens that underwent mechanical debridement (rubbing) exhibited exaggerated corrosive effect.

Similarly, a study done by Ericsson *et al*. using pure titanium implants showed acidic chemicals having a pH of less than 3 caused destruction of the titanium oxide layer causing discoloration, corrosion, pitting, and etching on the surface [91-112]. From these studies, a correlation between

pH and corrosion of titanium surface can be established [90, 113, 114]. Specifically, titanium has a higher vulnerability for changes in surface morphology when in contact with acidic substances because the acidic chemicals have a higher concentration of dissolved H^+ ions, these easily dissolute the titanium oxide layer [115].

Discoloration of titanium surface is the result of electrochemical attack; this happens when the surface gets oxidized. The titanium oxide layer in its native state has Ti^{4+} ions which are colorless. When the oxide layer is oxidized, it produces Ti^{3+} and Ti^{2+} ions. The Ti^{3+} oxidation state produces a characteristic purple color, while the Ti^{2+} produces a distinctive yellow discoloration on the surface as demonstrated in previous studies [90, 8]. The presence of yellow and purple discoloration on treated titanium surfaces in this study corroborate the conclusion of corrosion induced by the performed procedures.

Furthermore, the last part of the study evaluated the synergistic effects of bacterial adhesion and detoxification on growth of pre-osteoblasts on titanium surfaces. Growth and proliferation of osteoblasts on implant surfaces ensure the natural progress of osseointegration, which is a major contributor to the success of a dental implant. The concern with the detoxification method is that once debridement is done, the entire surface of a dental implant is cleaned and many times adhered bone-forming cells on the surface can be scrubbed off. Numerous studies have tried to evaluate re-growth of bone-forming cells on implant surfaces post-detoxification, but again results are mostly inconclusive provided the differences in experimental design [30, 37, 41, 84, 86].

Results from cell compatibility revealed chemicals used along with mechanical force had a considerable consequence on the proliferation and differentiation of pre-osteoblasts. In general,

cell viability was found to be on average lower on samples subjected to rubbing method in relation to samples subjected to immersion. In addition, when comparing to non-treated specimens (control), treated titanium surfaces exhibited lower cell viability and differentiation.

Although it was hypothesized that acidic chemicals would hinder growth of bone-forming cells, surprisingly, citric acid being the most acidic chemical included in this study, had the second highest cell viability and ALP activity for rubbing method, when compared to the other chemicals. The use of citric acid for detoxification has been well studied and the positive effect of this chemical revealed that it may increase the changes of new attachment of cells on root surfaces [116-119]. A study done by Alhag *et al.* in 2008 [120] and Kolonidis *et al.* in 2003[121] in dogs assessed three different treatment techniques: surface treatment with (1) supersaturated citric acid, (2) brushing with toothbrush, and (3) swabbing with hydrogen peroxide for 1 minute. All three techniques were followed by rinsing with saline. In both studies, it was concluded that new bone-to-implant contact was established, and that this was associated to an increase in surface roughness induced by the treatment.

The study conducted by Wheelis *et al*. revealed that almost all treatment methods (rubbing and immersion) using the chemicals investigated in this study caused an increase in surface roughness of titanium. Specifically, citric acid was seen to have the most distinct increase in roughness from immersion (approximately 5nm) to rubbing (approximately 25 nm) [89]. This is an interesting observation because *in vivo* studies have shown that in increase in dental implant surface roughness offers a more suitable anchorage surface for bone cells to adhere to the surface [122, 123].

When analyzing both cell viability and ALP activity, a trend that was observed was that the combination of mechanical abrasion with acidic chemicals initially induced low proliferation of bone-forming cells, but ultimately resulted in higher number of pre-osteoblast differentiation. On the other hand, samples that did not experience mechanical abrasion initially had high proliferation but did led to low differentiation rates. This trend was observed with the other chemicals as well, including chlorhexidine, doxycycline and saline. This observation disproved the initial hypothesis which stated that both proliferation and differentiation of pre-osteoblasts would be hindered.

From this observation, it was deduced that cells that were strongly adhered to titanium surface but in low populations were able to differentiate, while cells that were not adhered strongly were not able to differentiate. Since more differentiation was observed on samples that was subjected to mechanical abrasion, from this, it can be assumed that an increase in surface roughness ultimately lead to better growth of bone-forming cells. So it can be concluded from this study and supporting literature discussed above, that an increase in surface roughness can be attributed to the high cell viability observed with the citric acid-rubbing treatment performed in this.

In summary, this study demonstrated that bacteria can create suitable conditions for oxide layer damage. In addition to this effect, detoxification of contaminated titanium surfaces using acidic chemicals and mechanical forces also induced changes in surface morphology and oxidation state of titanium resulting in discoloration and pitting attack. So the combination of these two actions led to a significant change in morphology of the surface as hypothesized. This change in morphology did impact the cell behavior on treated titanium surfaces, but not as hypothesized. Although cell proliferation was low on samples treated with a combination of mechanical abrasion

and acidic chemicals, ultimately there was a higher differentiation on these samples compared to samples that did not endure mechanical abrasion. This lead to the presumption that pre-osteoblasts were able to differentiate into osteoblasts on surfaces that were roughened with the detoxification method. It can be inferred from this study that morphology of titanium surface plays a key role in cellular attachment and differentiation.

Some of the limitations of this study included contamination of pre-osteoblast cell line, which when encountered, the study was halted and started all over. In addition, titanium surfaces were re-polished more than two times to re-use in this study. Even though they were re-polished to mimic the original state, minor scratches and dents were still visible. Future studies will expand on the observations of this study by adding a mixture of the 3-steps included in this study and surface roughness measurements before and after bacterial adhesion and detoxification to look at how surface roughness changes with each method. Additionally, it is necessary to further investigate why bone-forming cells are more compatible on titanium surfaces subjected to mechanical abrasion.

4.6 CONCLUSION

In conclusion, it was observed, bacterial adhesion on titanium surface inflected severe discoloration and pitting. In addition, manual rubbing combined with acidic chemicals exacerbated this effect by producing more pronounced discoloration, which indicates drastic changes in the oxidation state of titanium. The most damaging treatment found in this study was rubbing with citric acid, and the least damaging was immersion with saline.

Combination of manual application of force (rubbing method) and acidic chemicals resulted in low proliferation rates which indicated cytotoxicity of treated titanium surfaces to pre-osteoblasts. Immersion in saline and doxycycline produced highest percentage of cell viability, while immersion with citric acid produced the lowest cell viability. In general, ALP activity of pre-osteoblasts was higher on samples treated by rubbing method than on samples treated by immersion method. Although pre-osteoblast proliferation was low for samples subjected to rubbing method compared to immersion; ALP activity was higher for rubbing than for immersion overall. It can be concluded that the combination of mechanical debridement with acidic chemical did hinder cell proliferation, but ultimately led to a higher differentiation of bone-forming cells, which indicated that surface morphology played a key role in cellular behavior.

CHAPTER 5

SUMMARY AND CONCLUSION

In vitro testing methodology was developed to examine surface morphology changes after bacterial adhesion and detoxification on titanium surface. In addition, pre-osteoblast cell proliferation and differentiation on titanium surfaces post-treatment was quantified. This was done by developing a 3 step procedure involving: (1) immersion of cpTi samples in a mixedacidic bacterial culture to contaminate the sample with peri-implantitis inducing bacteria; (2) detoxification of titanium using rubbing and immersion method combined with use of chemicals; and lastly (3) conducting a 7-day MTT and ALP assay on pre-osteoblasts that were seeded on treated sample surfaces. Surface analysis of bacteria contaminated titanium revealed significant yellow and blue discoloration in addition to pitting on sample surface. Similarly, titanium surface treated by rubbing method combined with acidic chemicals such as citric acid and doxycycline exhibited severe discoloration, whereas saline and chlorhexidine had minimal or not morphological changes.

Areas of discoloration after bacterial adhesion was evidence of titanium oxide layer oxidation due to the low pH of metabolites produced by bacteria. Likewise, low pH of acidic chemicals and application of mechanical force inflicted titanium oxide damage causing discoloration and pitting.

Rubbing and immersion treatment methods both had significant impact on proliferation and differentiation of pre-osteoblast cells. Though it was hypothesized rubbing in congruent with

acidic chemicals would hinder both proliferation and differentiation, evidently, it was observed to hinder cell proliferation but overall resulted in higher differentiation of pre-osteoblasts when compared to the immersion method. Further analysis of surface roughness and corrosion with the methodologies developed in this study will help to better elucidate why bone-forming cells are more compatible on titanium surfaces subjected to mechanical abrasion.

CHAPTER 6

FUTURE WORK

The work presented in this research study is a new approach developed to study the synergistic action of bacterial adhesion, detoxification and growth of bone-forming cells. This experimental setup has the versatility to accommodate different dental implant material as well as different peri-implantitis treatment methods. Therefore, this protocol can be applied to different types of material available in the market such as: zirconia, titanium-zirconium alloy and Ti6Al4V alloy surfaces. This would provide a good understanding of how each material reacts differently to the synergistic activity.

Another future project would be to use the same protocol of this study but to include different types of dental implants available in the market as mentioned in Table 2.1 in chapter 2. This would provide a more in-depth understanding of how commercially available dental implants are effected by bacterial adhesion and detoxification methods.

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