Osseointegration of Mini Dental Implants

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To my wife Minnie,

To my sons, Prithm and Hukam,

To my parents

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Abstract

Dental implant supported overdentures have been known to improve patient satisfaction and quality of life. Mini Dental Implants (MDIs) have several advantages over conventional implants. The major advantages are that 1. The surgery is minimally invasive, 2. Transmucosal placement is possible using a single pilot drill and 3. They can be loaded immediately. These also offer an alternative for patients with conditions that restrict them from being candidates for standard width dental implants. Despite these advantages, evidence of their potential for osseointegration and long-term success is lacking, and there are relatively few studies investigating the osseointegration of MDIs.

We hypothesized that there is no difference in the osseointegration potential of MDIs and standard-sized implants. To test this hypothesis, an *in vitro* and a randomized *in vivo* animal study were designed. From the *in vitro* investigation, we found that implant surface property may play a significant role in the ability of osteoblastic cells to form initial attachment and proliferation. Thus, we designed three *in vivo* experiments using a rabbit tibia model to compare MDIs and standard implants for their potential to osseointegrate at different time-points. We used three different methodologic approaches: In the first, a resonance frequency analysis was carried out; results indicated that there is no difference in stability between the MDI and comparator implants ($p<0.05$; Wilcoxon's matched pair's sign-rank test). In the second approach, a histologic study showed that there were no differences between the implant types in the amount of bone implant contact ($p>0.05$; Mann-Whitney). Finally, nanoindentation testing demonstrated that the mechanical properties of bone near and apart from the bone/implant interface were similar between the two implant types ($p > 0.05$; ANOVA). In summary, the evidence from this project suggests that MDIs offer similar osseointegration potential as commonly-used standard sized implants. Therefore, we

recommend that randomized clinical trials with long-term follow-ups be conducted to determine whether MDIs and standard sized implants will demonstrate similar osseointegration characteristics under function and in patient populations.

Résumé

Les prothèses dentaires soutenues par des implants sont reconnues pour améliorer la satisfaction et la qualité de vie chez le patient. Les Mini Implants Dentaires (IDM) ont plusieurs avantages par rapport aux implants conventionnels. Les principaux avantages sont 1 : La chirurgie est peu invasive, 2 : Le positionnement transmuqueux est possible à l'aide d'une perceuse pilote unique et 3 : Ils peuvent être placés immédiatement. Ces implants offrent également une alternative pour les patients avec des conditions qui les empêchent d'être des candidats pour des implants à taille standard. Malgré ces avantages, il manque la preuve de leur potentiel pour l'ostéointégration et le succès à long terme car il y a relativement peu d'études sur l'ostéointégration des IDM.

Nous avons fait l'hypothèse qu'il n'y a pas de différences dans l'ostéointégration potentiel des IDM et des implants de taille standard. Pour tester cette hypothèse, une étude *in vitro* et une autre étude *in vivo* d'un essai randomisé avec des animaux ont été conçues. À partir de l'étude *in vitro*, nous avons constaté que la propriété de la surface de l'implant peut jouer un rôle significatif dans la capacité des cellules ostéoblastiques de former l'attachement initial et de proliférer. Ainsi, nous avons conçu trois expériences *in vivo* à l'aide d'un modèle de tibia de lapin pour comparer les IDM et les implants standards sur leur potentiel d'ostéointegration à différents moments. Nous avons utilisé trois approches méthodologiques différentes : dans la première, une analyse de la fréquence de résonance a été effectuée ; les résultats ont indiqué qu'il n'y a pas de différences de stabilité entre les IDM et les implants de comparaison ($p < 0.05$; test de somme de rang de Wilcoxon). Dans la deuxième approche, une étude histologique a démontré qu'il n'y avait pas de différences entre les types d'implants selon la quantité de contact d'os sur l'implant ($p > 0.05$; Mann-Whitney). Enfin, les essais de nano-indenteur ont démontré que les propriétés mécaniques d'un os situé près

de l'interface de l'implant/os étaient similaires avec les deux types d'implants ($p > 0.05$; ANOVA). En résumé, les éléments de preuve de ce projet suggèrent que les IDM offrent des potentiels d'ostéointégration similaires aux implants communs de taille standard. Par conséquent, nous recommandons que des essais cliniques randomisés avec suivis à long-terme soient effectués pour déterminer si les IDM et les implants de taille standard feront la démonstration de caractéristiques d''ostéointégration similaires dans la population de patients.

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Thesis Outline

This doctoral thesis has been prepared as a manuscript-based thesis. This thesis is comprised of 6 chapters. Chapter 1 gives a brief description on Mini Dental Implants and the development of techniques for the measurement of osseointegration. Chapter 2 of the thesis covers the rationale and objectives of the study. Chapters 3 and 4 contain the four manuscripts that have been published/submitted for publication.

Chapter 5 offers a General Discussion, strengths and future directions for the research, and chapter 6 comprises the Conclusions.

Manuscripts presented in the Thesis Chapters 3 and 4 are as follows:

Chapter 3 ***In vitro study***
Part I- Comparing mini dental implants with standard implants: A Cell Culture Study

Manuscript 1
Title- **In vitro comparison of two titanium dental implant surface treatments: 3M™ESPE™ MDIs versus Ankylos®**
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Chapter 4 ***In vivo*** **animal study**

Part II- Measuring and comparing the stability of mini dental implants and standard implants by resonance frequency analysis.

Manuscript II
Title - **Customized SmartPeg for Measurement of Resonance Frequency of Mini Dental Implants**

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Part III- Comparing bone apposition on the surface of mini dental implants and on standard implants with histomorphometric methods.

Manuscript III
Title- **Osseointegration of Standard and Mini Dental Implants: A Histomorphometric Comparison**

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Part IV- Measuring the elastic modulus and hardness of the bone-implant interface in mini dental implants and standard implants with nanoindentation method.

Manuscript IV
Title- **Exploring the Mechanical Properties of Bone Surrounding Osseointegrated Mini Dental Implants and Ankylos® Implants using Nanoindentation**

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Contribution of Authors

This thesis includes four prepared manuscripts of which the candidate is the first author.

In all of the articles, the PhD candidate **Jagjit Singh Dhaliwal** made major contributions to the design and performance of experiments, execution of the technical procedures, data collection, data analysis and preparation of the manuscripts. In all of the manuscripts, all the co-authors played a significant role in the research.

Manuscript I- Jagjit Singh Dhaliwal conceived the study and drafted the manuscript. Juliana Marulanda carried out the cell cultures experiments, analyzed the data and drafted the manuscript. Sharifa Alebrahim established the *in vitro* culture system. Jingjing Li generated and characterized the BMP-2-transfected cell line, Prof. Jocelyne Feine participated in designing the study. Dr. Monzur Murshed provided lab support, designed and coordinated the study, analyzed the data and drafted the final version of the manuscript. All authors read and approved the final manuscript.

Manuscript II- Jagjit Singh Dhaliwal carried out the experiments, collected data and drafted the manuscript, Dr. Rubens F. Albuquerque Jr. conceived the study and helped in revising the manuscript, Dr. Ali Fakhry contributed to the designing of the SmartPeg, Prof. Sukhbir Kaur provided laboratory support, and Prof. Jocelyne Feine supervised, participated in this study's design and overall coordination. All authors read and approved the final manuscript.

Manuscript III- Jagjit Singh Dhaliwal designed and carried out the experiments, collected and prepared the samples and drafted the manuscript, Dr. Rubens F. Albuquerque Jr. helped in designing of the study and revised the manuscript, Dr. Monzur Murshed provided support and access to his laboratory and shared writing of the document and Prof. Jocelyne Feine supervised

the study, overall coordination and edited the manuscript. All authors read and approved the final manuscript.

Manuscript IV- Jagjit Singh Dhaliwal designed and performed animal surgeries, collected and prepared the samples and drafted the manuscript, Dr. Rubens F. Albuquerque Jr. helped in designing of this experiment, Dr. Etienne Bousser provided laboratory support and helped in reviewing the manuscript, Dr. Thomas Schimtt conducted nanoindentation procedure and Prof. Jocelyne Feine supervised the study, overall coordination and edited the manuscript. All authors read and approved the final manuscript.

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List of abbreviations

MDI	Mini Dental Implant
NDI	Narrow diameter Implant
BMP	Bone Morphogenetic Protein
FBS	Fetal Bovine Serum
ALPL	Alkaline Phosphatase
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle Medium
SEM	Scanning Electron Microscope
BIC	Bone Implant Contact
ISQ	Implant Stability Quotient
RFA	Resonance Frequency Analysis

Chapter I: Introduction

1.1 History of Dental implants and Osseointegration: It was discovered in the 1930s through archaeological excavations in Honduras that the Mayan civilization had used dental implants (3). A fragment of mandible with implants made of pieces of shells was found dating from about AD 600 and replacing three lower incisors. Compact bone was also found around two of these implants. The present dental implant story began during World War II when Dr. Norman Goldberg, in his army service, considered dental rehabilitation with the help of metals that were already being used for replacing other parts of the body (1). In collaboration with Dr. Gershkoff, he created the first successful subperiosteal implant in 1948. This was the very foundation of implant dentistry, and they became the first individuals to teach implant techniques in dental schools (1).

In 1960s the term “osseointegration” was first introduced to explain the phenomenon for stable fixation of titanium to bone by the Swedish orthopedic surgeon, PI Brånemark. He discovered that bone can form around titanium and an effective union can take place between bone and titanium without rejection (2, 3). Brånemark termed it as "Osseointegration", and it was defined as the direct contact between the surface of an implant and the surrounding bone (4). While the term "functional ankylosis" was used by Schroeder et al in 1981 (5), in 1993 Albrektsson and Zarb (6) defined osseointegration as "a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved, and maintained in bone during functional loading". The introduction of osseointegrated implants was a major scientific discovery, resulting in a new era in oral rehabilitation.

Dental implants have been widely used for the stabilization of complete dentures and also help to maintain bone, function, esthetics, and phonetics and improve oral health related quality of life (7). Dental implants are available with different surfaces and sizes. The size of the dental implants usually ranges between 3mm (narrow) and 7 mm (wide) in diameter, depending on the size of the

bone into which the implant will be surgically inserted. The majority of implants placed worldwide fall within a "standard diameter" range of 3.7 mm to 4.0 mm (8). The implant length ranges between 6-20 mm (3). However, average length of most commonly used implant ranges from 8-15 mm; length is also dependent on the available bone (3).

1.2 Implant materials: A biomaterial that is used as an implant is supposed to demonstrate favorable tissue response and be highly biocompatible. The other desirable properties are high resistance to fatigue, high mechanical strength, low modulus of elasticity and superior wear resistance (9). It is challenging to find all these properties in one material. However, titanium and its alloy Ti-6Al-4V are desirable materials for the fabrication of implants owing to these properties, including a comparatively low inertness, hypoallergenicity, stiffness and weight, compared to other metals. They are also corrosion resistant in an *in vivo* environment and used in pure or alloy form for several present day implant designs (9).

The alloy is composed of 6% aluminium and 4% vanadium (Ti-6Al-4V). The heat treatment of these alloys enhances mechanical and physical properties, making them superb implant materials (10). The alloying elements to titanium produce additional properties. Aluminium stabilizes the α-phase, and vanadium stabilizes the β-phase. This lowers temperature of the transformation from α to β. The alpha phase encourages a good weldability, superior strength characteristics and oxidation resistance. Vanadium as a β-stabilizer maintains the higher strength of the beta-phase below the transformation temperature, resulting in a two-phase system (10). The elastic modulus of these materials is around 110 GPa (9). A β stabilized alloy contains vanadium, molybdenum, iron, chromium & zirconium and has greater tensile and yield strength than all α-alloys. Ti-6Al-4V is one of the best α-β alloys, as it can boast a combination of strength and stiffness and is resistant to corrosion. Ti6Al-4V ELI is used for many medical and dental implants due to its superb

biocompatible nature. ELI stands for “extra-low interstitial” version of Ti6Al-4V with lower specified limits on iron and interstitial elements C & O, and is an alpha + beta alloy. ELI grade alloy has excellent fracture toughness, fatigue crack growth rate and better mechanical properties at cryogenic temperatures as compared with a standard grade Ti6Al-4V alloy (11). Many studies have been conducted to determine the survival rate of dental implants, and a success rate of over 90% has been reported (12-15).

1.3 Surface Properties of Implants: It has been shown that surface chemistry and topology of these surfaces play a major role in their success or failure. Properties of the biomaterials which affect their relationship with cells are wettability, texture, chemistry and surface topography (18). Surface wettability is basically the surface energy, which affects the level of connection with the biologic environment (19). When exposed to a biological environment, titanium quickly forms a surface oxide (TiO2) which is a passivating layer. This layer acts as a protective barrier and remains attached to the surface of implant. The oxide layer may be responsible for the high biocompatible nature of the metal (16), offering a favorable interface on which osteoblastic cells can deposit bone and mineralize (9, 17). The oxide layer undergoes hydroxylation in the biological environment. This initiates wettability by water and communication of the surface with water shell surrounding protein biomolecules. This will lead to reduction in the time required for healing thereby providing a conducive interface and augmenting deposition of mineralizing bone around the implants and osseointegration (17). Therefore, the surface properties of implant materials are vital to the response of cells at the interface influencing the growth and quality of newly formed bone tissue (18, 20).

1.3.1 Techniques for Alteration of Implant Surface: Initial implant surface was the machined implant surface design which required many months for osseointegration. A range of techniques have come into being for creating a rough surface and enhance osseointegration of dental implants. Various methods for altering the surface include plasma spraying, sandblasting, acid etching and oxidation. The modification techniques may be either additive or subtractive of the machined surface. The additive methods include plasma spray or hydroxyapatite (HA) coatings. The subtractive methods include sandblasting and acid etching. The implant surfaces are struck with particles of Silicon Carbide (SiC), Aluminium Oxide (Al2O3), glass, or Titanium Oxide (TiO2). Therefore, the process of abrasion with these particles produces a rough surface (21). The amount of abrasion is dependent on the size of the particles, medium, time and pressure of blasting, as well as distance of the implant surface to the particles source (22). The blasted surfaces can be further treated with acids to remove any residue from the surface and produce etched pits on the surface. Consequently, acid treatment will enhance roughness on the implant surface. Hydrofluoric, nitric and sulfuric acids are the most commonly used etching agents. The implant is immersed into the solution leading to erosion by creating microscopic pits on the surface (22). In addition to the mechanical methods, various chemical modifications e.g. the use of calcium, magnesium and fluoride ions have been explored (23). The use of osteoinductive agents like growth factors and BMPs has also been studied. It is thought that these agents can lead to osteoblastic cell differentiation helping in quicker bone formation and a solid bone implant interface (24).

1.3.2 Surface Roughness and Osseointegration: The degree of bone formation on an implant surface is due to three processes, which are osteoconduction, osteogenesis and osteoinduction. It has been established that alteration of the topographic configuration of implant surface enhances the bone-implant contact and early interaction at the interface. Alterations of implant surfaces may

influence the amount of bone formation at the bone implant interface by any or all of these processes (25, 26). Rougher surface implants have been extensively used and taken the place of machined surfaces in clinical uses and roughness in the range of 1-2 µm is favorable for osseointegration (27). Increased surface roughness will lead to enhanced surface area of the implant adjoining bone, better cell attachment on the surface of implant, higher amount of bone at the implant surface, as well as increased biomechanical interaction of bone and implant (28). It has been shown that compared with machined surfaces, roughened implants had a longer survival percentage (29).

Gotfredson et al. concluded that implants blasted with TiO2 particles displayed a considerably higher percentage of bone-implant contact (BIC) than titanium implants with a machined surface. A significantly higher removal torque was needed to unscrew the TiO2-blasted implants (30). Similar findings were observed by Ericksson et al. (31). Comparison of removal torque of two different surface textures of screw-shaped CPTi implants in rabbits showed that rough surface implants had significantly higher removal torque than the smooth surface implants, after 6 weeks of healing (32). In another animal study by Wennerberg et al., implants of three different surfaces were inserted in rabbit tibia. Significantly higher percentage of BIC and removal torque values were observed in implants blasted with TiO2 and Al2O3 compared to machined implants after 12 weeks of healing (33). In another study, implant surfaces prepared by machining, blasting with TiO2 particles, and acid etching were compared. The authors concluded that acid etched surface implants withstood counter torque forces more effectively (34).

1.4 Mini Dental Implants: A large body of literature recommends the use of mini dental implants for stabilization of removable partial and complete dentures in selected situations. The 3M™ESPE™ Mini Dental Implants (MDIs) were introduced on the market; the system makes use

of a self-tapping threaded screw design and needs minimal surgical intervention. These implants are fabricated from Ti 6Al-4V ELI titanium alloy (11). Mini dental implants or smaller implants are being widely used for stabilizing complete dentures (35), orthodontic anchorage (36-38), single tooth replacements (39, 40), fixation of surgical guides for definitive implant placement (41) and as transitional implants for the support of an interim removable prosthesis during the healing phase of final fixtures (42, 43). These have become increasingly popular in many countries for denture stabilization. The MDIs have many advantages over the regular implants used for overdentures.

The surgical protocol of MDIs is different and simpler than with regular implants (39), with the surgery being minimally invasive compared to conventional full-flap implant surgery. Incisions and flap reflections are not required and transmucosal placement is possible using a single pilot drill. This helps in reducing post-operative discomfort and minimizing resorption of bone during healing (44). The flapless method helps to prevent disturbance of blood supply to the bone. It has been shown that bone healing around immediately loaded transitional implants is not disturbed and causes no bone loss (45). The need for sutures or long recovery periods is eliminated, and they can often be loaded immediately.

Using these implants, the patient can walk into the office in the morning and leave on the same day with a full set of teeth and is even allowed to eat on the same day. These implants can work well for patients with significant bone loss that restricts them from being a candidate for standard width dental implants. They are also a solution for patients who have ridge deficiency and who cannot have surgery for medical reasons (46). Mini dental implants are also cost effective, with the price of one MDI being 3.5 times lower than that of a standard size mandibular implant (Nobel Biocare SteriOss Implant) (47), resulting in significant cost savings.

Various authors have stressed the importance of biomechanical factors such as type of loading, the bone-implant interface, the length and diameter of implants, the shape and characteristics of the implant surface, the prosthesis type, surgical technique, patient age, gender as well as the quantity and quality of the surrounding bone in the success of implants (48-51). The stability of the dental implants seems to play a major role as well, comprising primary stability (stability immediately after insertion) and secondary stability (obtained due to osseointegration) (52). The reasons for failure of implants are poor oral hygiene, poor bone quality, compromised medical status of the patient and biomechanical factors (53, 54).

Ultimately, the success of these implants will depend on their union with the surrounding bone. Relevant literature shows that studies have been attempted to measure the osseointegration of implants. However, there is considerable confusion in the literature regarding the best method to monitor the status of a dental implant.

1.5 Cell Culture Models: A literature search reveals that cell culture models have been frequently used to examine the response of osteoblastic cells on different implant surfaces. Comparative studies show the effects of various surfaces on cellular phenotypes. Osteoblastic cell attachment, morphology, viability and differentiation on different types of implant surfaces for example mirror-polished (Smooth-Ti), alumina-blasted and acid-etched (Alumina–Ti), SLA (sandblasted, large-grit, acid-etched; supplied by Straumann AG) as well as biphasic calcium phosphate grit-blasted and acid-etched (BCP–Ti) titanium have been studied. It was concluded that all of these surfaces were cytocompatible. A similar osteoblastic cell behaviour was observed on BCP-blasted and SLA surfaces (21).

A number of studies suggest that composition, roughness and surface energy of the implant influence initial attachment and dissemination of osteoblastic cells (55-58). Some studies have

reported that attachment, distribution and proliferation were faster on smooth surfaces than rough surfaces; however, differentiation was augmented on rough surfaces (56, 59-61). Dual acid etched implant surfaces seem to augment the attachment process of osteogenic cells and fibrin which leads to formation of bone on the surface of the implant (62).

1.6 Animal Models: *In vitro* approaches with cell or tissue cultures can be used initially to test a new material to prevent unwarranted use of animals. However, it may not be adequate to ascertain whether the material is biocompatible and safe in human beings. In the process of development of new materials including dental and orthopedic implants, it is essential that these materials be evaluated in animal models before their use in humans (63). A number of factors influence the selection of animal species for a particular study, namely, the cost (acquiring and caring), availability, ethical issues, tolerance to captivity, acceptability to society and ease of housing (64). The animal species commonly being used are rodents, rabbits, pigs, sheep, goats and dogs, with varying advantages and disadvantages. For instance, there may be ethical issues in the use of companion animals such as dogs, while other issues that may arise range from availability to housing and handling (63). To illustrate, rabbits are easy to handle compared with other animals due to their temperament and size and many are able to be kept together for easier simultaneous observation (65). Rabbits are also more easily available and less expensive compared to large animals (66). Additionally, rabbits' bones are large enough for insertion of several implants which is not possible in rats (63). The number of animals required for a particular experiment can also be reduced as they can serve as their own controls (67). New Zealand white rabbits in particular rapidly attain skeletal maturity by 28 weeks of age, which is highly suitable for experimental studies (68), and their long bones consist of primary bone tissue which heals quicker. Consequently, it takes six weeks for an implant to be osseointegrated in rabbits as opposed to three

to four months in humans (69). In addition, the recommendation is only six implants per rabbit as per international standards for biological evaluation of medical devices (ISO 10993-6:2007) compared to twelve for larger animals. Considering all the advantages, rabbits seem to be a good model for testing the implants.

1.7 Methods for Evaluation of Osseointegration:

Various techniques have been used for the assessment of osseointegration to study various implant designs and materials. These mainly include histomorphometric evaluation, biomechanic evaluation (Pull out and Push out tests and Removal Torque measurements) and stability measurements.

The following literature review shows various methods that have been used to demonstrate the osseointegration potential of dental implants.

1.7.1 Biomechanical testing: Mechanical tests for the assessment of osseointegration mainly measure the degree of force required to cause shear disconnection of the implant surface and peri-implant bone. The degree of force required for removal are noted several times and compared to assess the effects of surface characteristics of implants on osseointegration. The quality of osseointegration is indirectly calculated from these measurements. The Brånemark group has studied the mechanical properties of osseointegration through torsion tests, pull out tests and lateral loading tests (70-72). Many *in vivo* implant studies (73-81) have been conducted to measure the mechanical interface of implant and bone in various ways.

1.7.1.1 Pull-Out Tests: These tests are used to evaluate the shear failure load of bone when a tensile force is applied on the long axis of the implant and the peak force prior to failure is recorded with an Instrom machine. Kraut et al. (82) described a "pullout" test, though useful in delineating a time-dependent increase in resistance to pull-out force, it may not be directly applicable to the

question of torsional resistance as applied in clinical treatment protocols. These tests necessitate precise orientation of the implant towards the direction of the force to prevent unwanted force application (83). Fan et al. evaluated the effect of mechanical loading on the osseointegration with a pull-out test between the loaded and non-loaded implants (84).

1.7.1.2 Push-Out Tests: This test is also performed with an Instrom machine. The test measures vertical loads on a bone-implant sample positioned on a supporting jig. The coronal and apical ends of the implant should be free of bone. The force is applied on the coronal end and apical end which is exposed and should allow smooth extrusion of the implant from the bone. The machine is used to direct force on the implant and the peak force which represents loosening of the implant is noted down (85). The test results may be affected by distance between the implant and supporting jig and elastic modulus of the implant (86).

1.7.1.3 Removal Torque Test: This test has been used to study the osseointegration of threaded dental implants (81). The removal torque is measured with a torque gauge instrument connected to an implant-bone specimen. The maximum torque required to remove the implants is documented. It provides an indirect value of the shear force needed to rupture the bone-implant interface (32). Carlsson et al. compared the ability to resist removal torque of rough surface vs. smooth surfaced implants after six weeks of healing in the rabbit model (32). The measures of the implant-bone interaction may help to distinguish between groups. However, the clinical significance of the findings in these studies is unknown.

1.7.2 Stability Testing: A non-invasive and clinical test for the osseointegration of dental implants is the absence of mobility and sufficient level of bone around the implant measured by radiographs. The non-invasive methods for stability testing include Periotest and Resonance Frequency Analysis (87-91). Some authors have suggested that primary stability is a more important factor in

the long term success of the implants than other factors such as quality and quantity of the surrounding bone. Researchers have studied factors affecting the stability of the implants. Therefore, it seems that primary stability is a critical factor to predict whether or not the implant will be successful. It is said that micro movements of implants at an early stage are important for primary stability (52, 92). According to Szmukler et al. (93), micro movements induced by early loading of mini-implants are detrimental to osseointegration. Resonance Frequency Analysis is a quantitative method used to assess implant stability. The first studies using Resonance Frequency Analysis were published in 1996 (94). The Osstell ISQ instrument was launched in 2000 after the study by Meredith et al (92). The Implant Stability Quotient (ISQ) was developed converting kHz units to ISQ on a scale of 1-100. Increases in ISQ measurements are a measure of improved bone stiffness and healing around the implant, with a higher value indicating better stability. The Osstell ISQ device is a type of an electronic tuning fork which converts kHz to ISQ automatically, and measures sound waves generated by the unit through the implant body by way of a rod (SmartPeg) connected to the implant. These SmartPegs are company specific for standard diameter implants. A number of studies have been performed on regular implants on Resonance Frequency Analysis (90, 95), which has been used to document changes in the bone healing along the implant bone interface by measuring the stiffness of the implant in the bone tissue (96-99). It has also been used to determine whether implants are ready for the final restoration (100) or to be loaded (98), as well as to identify the implants at risk (101, 102). There are no published studies on the ISQ measurement of single piece Mini Dental Implants, as SmartPegs for these implants are not available to date. These are one piece implants and do not have an internal thread for the SmartPeg attachment. A custom made SmartPeg can be fabricated to facilitate measurement of ISQ for these implants.

1.7.3 Bone Implant Contact (BIC): The percentage of implant surface in contact with bone on a microscopic level is called Bone to Implant Contact (BIC). Bone-titanium interface structure was described by Sennerby et al (103, 104). They observed the healing process (3 days post insertion) around screw-shaped implants of commercially pure titanium in rabbit cortical bone. The process is initiated with a hemorrhage which fills the entire interface. Osteoid producing osteoblasts were seen at the endosteal surface and migration of mesenchymal cells and macrophages from the marrow took place. Bone formation was first detected on 7th day on the endosteal surface of the original cortex as a lattice of trabecular woven bone close to the implant surface. The woven bone serves as a foundation for the creation of an osteoid layer. The quality of the tissue, both mechanically and metabolically is influenced by remodelling of woven to lamellar bone (105). In due course, these two types of bone blend and fill the implant threads, with bone-titanium contact and bone area in the threads improving up to 6 months post insertion of implants.

A common method to evaluate biological responses to an implant is measurement of bone-implant contact, referred to as histomorphometry at the light microscopic level. In evaluating the integrated state of an implant, a quantitative measure of bone contact is compared to the relative strength that the implant has when one attempts to remove it. Bone to implant contact is one of the parameters which has been used extensively to study the amount of bone apposition next to the implants (106-112). The examination of histologic specimens for calculating the BIC percentage is considered as a reference criterion for establishing the degree of osseointegration of an implant (79). Whenever an implant is inserted in the jaw, it is in contact with compact and cancellous bone and, commonly, there is a significant amount of variation in mineralized bone-to-implant contact length alongside the implant surface. In animal studies, Deporter et al. reported large differences in contact length fractions in the coronal, middle and apical regions. These were observed under different loading

conditions (113, 114). Subsequently, Johansson and Albrektsson highlighted that the amount of bone in direct apposition to the implant surface is essential for mechanical retention (76). In a comparative study, it was shown that a hydrophilic sandblasted and acid etched SLA implant surface had greater Bone Implant Contact (BIC) than a regular SLA surface (115).

1.7.4 Micro Computed Tomography (Micro CT): This is a non-destructive method for viewing the interiors of an object and can also be used for analysis of bone microstructure. It also does not require complex procedures for preparation of specimens for microscopy (116). It is important to note that bone implant interface is dynamic and three-dimensional. The percentage of BIC alters continuously due to the dynamics of the bone (117).

Micro CT analysis has been shown to provide morphological and architectural properties of bone. It has been used to study bone implant contact from three-dimensional reconstruction images (118-120). This method provides information on properties such as sponginess, bone density and morphology. The parameters measured are bone volume, bone surface, trabecular thickness, trabecular separation and bone connectivity (121). The information on bone architecture from Micro CT analysis has been shown to be closely related to mechanical properties of bone tissue (122). The results obtained by Micro CT on Bone Implant Contact have been comparable to the standard histology sections (123), but there are possibilities of producing artifacts in Micro CT images due to the metallic nature of implants. The causes for this may be beam hardening by x ray spectrum dispersion, photon starvation and poor signal to noise ratio as well as high contrast between the metal and adjacent structure (124).

1.7.5 Mechanical Properties Assessment: A high elastic modulus (Young's modulus) in any material suggests high material stiffness. There is a limited amount of literature studying biomechanical properties of bone surrounding the dental implants. Greater bone mass may not

always indicate higher bone strength. Therefore, it is vital that mechanical properties of bone are measured. Nanoindentation of bone around implants can possibly explain the qualitative aspects of osseointegration (125). However, there are few studies advocating the use of nanoindentation tests for measuring the elastic modulus and hardness of bone around the implants at the micro structural level. Studies have been conducted to examine the mechanical properties of the individual constituents of bone, such as the lamellae and the osteons of the bone surrounding the dental implants. The indentations can be performed at the bone implant interface for studying the bone quality (126-129). There is also limited literature on the biomechanical properties (especially hardness/elastic modulus) of bone integrated to mini implant surfaces.

1.8 Need for the Study: The osseointegration potential of the 3M™ESPE™ MDIs has not been studied. New implant systems entering the market must be studied *in vitro* and *in vivo* with animal models to demonstrate their osseointegration capability and potential success in humans. A literature search was performed and no published studies in animals or humans were found from the databases. Most of the research directed towards mini implants is for orthodontic purposes. However, orthodontic forces are normally unidirectional and constant, unlike occlusal forces. Despite the advantages of mini dental implants, evidence on their potential for osseointegration and long term success is lacking.

A major strength of this research is that a variety of methods were used to thoroughly explore and measure osseointegration of the 3M™ESPE™ Mini Dental Implants on the same implant samples to maintain consistency of results. An *in vitro* cell culture experiment was performed first to study osteoblastic cell adhesion, proliferation and differentiation on test and experimental implant surfaces. Since it is not possible to replicate the dynamic *in vivo* environs involving the bone-implant interactions in cell cultures, it was important to perform an animal study using the same

comparator surface to substantiate the results. Many factors may impact osseointegration; therefore, it may be necessary to evaluate as many parameters as possible in the same samples in order to understand bone healing around implants as opposed to individual investigations on a variety of samples.

Thus, we have designed a series of studies using a variety of methods to thoroughly explore the osseointegration of the 3M™ESPE™ Mini Dental Implants; the results will assist in understanding treatment selection, prognosis and outcomes for patients.

Chapter two: Rationale, research hypothesis, and objectives

2.1 General aim:

To test the hypothesis that there is no difference in the osseointegration of Mini Dental Implants (MDIs) compared to commonly-used standard sized implants.

2.2 Rationale of the study:

Considering the advantages of MDIs over standard implants for mandibular overdentures, it is important to establish their osseointegration capacity. Newer implants and materials must be studied with *in vitro* models first, followed by animal and human studies. Therefore, a series of experiments were designed to assess the osseointegration potential of 3M™ESPE™ MDIs *in vitro* and *in vivo*. The first study was conducted *in vitro* comparing the adherence, proliferation and differentiation of osteoblastic cells on the MDI surface with a standard implant surface. Consequently, *in vivo* studies were designed to investigate the osseointegration potential of these implants using an animal model. The *in vivo* experiments included a Resonance Frequency Analysis (RFA) with a newly developed customized SmartPeg for MDIs, a histological study and measurement of mechanical properties with the nanoindentation method. These approaches were used, stage by stage, to measure the osseointegration potential of MDIs. We developed a customized SmartPeg for these single piece implants, as it is not possible to measure their stability non-invasively with the devices currently available on the market. Histological methods are regarded as the "gold standard" for assessing bone formation adjoining implants. The nanoindentation method was used to measure mechanical properties of implant material and surrounding bone. We sectioned each implant embedded in resin block into two parts: one half was used for histomorphometry and the other for depth-sensing nanoindentation tests.

2.3 Hypothesis: The null hypothesis for purposes of this research is that there is no difference in the osseointegration of Mini Dental Implants (MDIs) compared with Ankylos® implants in the rabbit tibia.

2.4 Objectives: The specific objectives of this research were:

1. To study the adherence, cell proliferation and differentiation of bone morphogenetic protein 2 (BMP2)-treated C2C12 myogenic cells and MC3T3-E1 preosteoblasts on two types of implant disk surfaces: 3M™ESPE™ MDI-sandblasted and passivized (Test group) and Ankylos®- sandblasted and acid etched (Control group) *in vitro*.
2. To measure and compare the stability of 3M™ESPE™ MDIs and regular implants by resonance frequency analysis.
3. To compare bone apposition on the surface of 3M™ESPE™ MDIs and on standard implants by means of histomorphometric methods.
4. To measure the elastic modulus and hardness of the bone implant interface in 3M™ESPE™ MDIs and standard implants with the nanoindentation method**.**

2.5 Ethics approval: The study protocol was approved by the Institutional Ethics Review Board (IRB) vide **Animal Use Protocol # 2012-7221** with McGill University and its Affiliated Hospitals' Research Institutes for the project.

Chapter three: *In vitro* Study

3.1 Comparing Mini Dental Implants with Standard Implants: A Cell Culture Study

Successful osseointegration implies close contact of bone with the surface of an implant. Recently, there has been interest in immediate loading protocols in dental implants. However, the response rate of bone formation depends on a favourable implant surface. The implant surface chemistry and roughness have a key role in the biological events that ensue after implantation (115, 130). The surfaces that are currently available on the market range in thickness from nanometers to millimeters. There can be three degrees of topographical features like macro, micro and nano sized. Surface treatment techniques are applied to enhance the quality and quantity of bone to accelerate healing (131). Modification of surfaces seems to augment the chances of early osseointegration (131). Several studies have shown that, compared with a smooth surface, a rougher surface provides enhanced long term mechanical strength and early fixation of the prosthesis (30, 33, 132). A number of implants with an array of surface properties are available commercially. The response of osteoblastic cells on implant surfaces can be examined using cell culture models. With the help of these models, researchers can examine the growing ability, adhesion, morphology, proliferation and differentiation of osteoblastic cells on implant surfaces with different compositions and topologies.

It has been shown by a number of researchers that 1-10µm of surface roughness increases the connections between the implant surface and bone (30, 33, 132, 133). Implants with rough surfaces have also shown improved clinical results compared with smooth surface implants (29).
Hydrophilic surfaces have been shown to be more advantageous compared with the hydrophobic surfaces because of superior interaction with biological fluids (134). A number of studies have been conducted using *in vitro* models of osseointegration.

The surfaces of 3M™ESPE™ MDIs are treated to impart roughness which includes sandblasting with aluminium oxide particles, followed by cleaning and passivation with an oxidizing acid. The treatment process leads to a moderate roughness of 1–2 μm on the implants (135).

The Ankylos® implant has the FRIADENT plus surface (Dentsply Implants, Mannheim, Germany). It is formed by sandblasting in a temperature controlled process and acid etching (hydrochloric, sulfuric, hydrofluoric, and oxalic acid) followed by a proprietary neutralizing technique. The mean surface roughness caused by the process is approximately 3.19 μm (136).

Mini Dental Implants for overdentures have been recommended for immediate loading/early loading. Therefore, it is important to know whether the surface is conducive for osseointegration compared to a standard well-established implant surface, such as that on the FRIADENT plus implant. However, the literature does not show sufficient evidence regarding MDIs on whether these implant surfaces are as good as standard sized implants for osteoblastic cell adhesion and bone formation. The following manuscript is under revision with the International Journal of Implant Dentistry.

3.2 Manuscript I

In vitro comparison of two titanium dental implant surface treatments: 3M™ESPE™ MDIs versus Ankylos®

Running title: Cell culture on surfaces of 3M™ESPE™ MDI and Ankylos®

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Abstract

Background: The objective of this study is to compare the proliferation and differentiation of osteogenic/osteoblastic cells on Ankylos® and 3M™ESPE™ MDI implant surfaces. In the current study, we hypothesize that there is no difference in the proliferation and differentiation capacity of osteoblastic cells when cultured on 3M™ESPE™ MDIs and standard (Ankylos®) implants.

Methods: Cells were grown on disks made of the same materials and with same surface texture as of the original implants. Disks were sterilized and coated with 2% gelatin solution prior to cell culture. C2C12 pluripotent cells treated with 300 ng/ml bone morphogenetic protein-2 (BMP-2) and a stably-transfected C2C12 cell line expressing BMP-2 were used as models for osteogenic cells. The Hoechst 33258 -stained nuclei were counted to assay cell proliferation, while alkaline phosphatase (ALPL) immunostaining was performed to investigate osteogenic differentiation. MC3T3-E1 cells were cultured as model osteoblasts. The cells were differentiated and assayed for proliferation and metabolic activities by Hoechst 33258 staining and Alamar blue reduction assays, respectively. Additionally, cultures were stained by calcein to investigate their mineral deposition properties.

Results: Electron microscopy showed greater degree of roughness on the MDI surfaces. Nuclear counting showed significantly higher number of C2C12 cells on the MDI surface. Although immunostaining detected higher number of ALPL-positive cells, it was not significant when normalized by cell number. The number of MC3T3-E1 cells was also higher on the MDI surface and accordingly these cultures showed higher Alamar blue reduction. Finally, calcein staining revealed that MC3T3-E1cells grown on MDI surfaces deposited more minerals.

Conclusion: Although both implant surfaces are conducive for osteoblastic cell attachment, proliferation and extracellular matrix (ECM) mineralization, cell proliferation is higher on MDI surface, which may in turn facilitate osseointegration via increased ECM mineralization.

Keywords: Cell culture, Osteoblasts, Implant surface

Introduction

Prosthetic devices are often used as surrogates for missing skeletal and dental elements. These devices are in close contact with the surrounding tissues and their functionality and stability are critically dependent on the successful integration within the tissue's extracellular matrix [ECM]. The surface of the implanted device directly interacts with cell and extracellular milieu and influences their biological activities affecting the healing of the implant site after the surgery, tissue regeneration and the formation of an organic interface with cells and ECM proteins.

Dental implants are a commonly used treatment for replacement of missing teeth and the long-term success of these implants depends on their proper integration with the mineralized bone, a process commonly known as osseointegration (1).

It has been a long-standing challenge to achieve their successful osseointegration in older population with poor bone mass and low bone turnover rates. Therefore, an ideal implant should have a surface which is conducive to osseointegration regardless of the implant site, bone quality and bone quantity. A large body of literature recommends the use of mini dental implants for stabilization of removable partial and complete dentures in selected situations (2). The 3M™ESPE™ Mini Dental Implants (MDIs) system makes use of a self-tapping threaded screw design and needs a minimal surgical intervention. Also, small size implants have been widely used for orthodontic anchorage (3-5), single tooth replacements (6, 7), fixing the surgical guides for definitive implant placement (8) and as transitional implants for the support of interim removable prosthesis during the healing phase of final fixtures (9, 10). The MDIs have several advantages over the regular implants used for overdentures such as; simpler surgical protocol and minimally invasive surgery, and they can often be loaded immediately (6). This helps in reducing post-operative distress to the patient and minimizing resorption of bone during healing (11). It has been

shown that bone healing around immediately loaded transitional implants is not disturbed and causes no bone loss, which represents a solution for patients who have ridge deficiency and that cannot have surgery for medical reasons (12, 13). Mini dental implants are also cost effective, the price of one MDI is 3.5 times lower than that of a standard size mandibular implant (14).

Most of the research is directed towards mini implants for orthodontic purposes. However, orthodontic forces are normally unidirectional and constant, unlike occlusal forces.

Despite the advantages of the MDI, evidence on their potential for osseointegration and long term success is lacking. (15-18). Newer implant systems entering the market must be studied first *in vitro* and then *in vivo* with animal models followed by human studies to demonstrate their osseointegration capability.

Modifications of implant surface properties have been shown to have a positive influence on its successful osseointegration (19-22). Surface properties such as roughness, topography and chemistry are strongly related to the biocompatibility of implants (23). Thus, modulation of these properties can be useful means to improve implant osseointegration in patients with poor bone quality. The most common treatments used for implant surface modifications are acid etching and sandblasting (24-27). Implants with moderate surface coarseness demonstrate a better bone response than a smoother or rougher surface (28-30). When an implant is placed in the bone, a series of cell and matrix events take place. These mainly include host response to the implant material and behavior of the implant in the host tissue, which culminates in intimate deposition of new bone on the implant surface (31).

The immediate event after implantation is adsorption of proteins (31). Various studies show that direct osteoblast-implant interactions are critical for proper osseointegration. Cell culture models are being commonly used to study bone-biomaterial interface using osteoblastic cells (32).

The surfaces of 3M™ESPE™ MDIs are treated to impart roughness which includes sandblasting with aluminium oxide particles, followed by cleaning and passivation with an oxidizing acid (33). The Ankylos® implant has the FRIADENT plus surface (Dentsply Implants, Mannheim, Germany). It is formed by sandblasting in a temperature controlled process and acid etching (hydrochloric, sulfuric, hydrofluoric, and oxalic acid) followed by a proprietary neutralizing technique (34).

In the current study, we examined the proliferation and differentiation characteristics of multipotent C2C12 cells and MC3T3.E1 preosteoblasts on 3M™ESPE™ MDI (Test group) and Ankylos®. The Ankylos® implant surface was used for comparison as it is a well-established and widely characterized standard implant. Thus, we hypothesize that there is no difference in the proliferation and differentiation capacity of osteoblastic cells when cultured on 3M™ESPE™ MDIs and standard implants.

Materials and Methods

Implant disks: Titanium disks made up with the same materials and surface characteristics as the original implants were obtained from the respective manufacturers. Two types of disks were used; small disks represented 3M™ESPE™ MDI implants, while the large disks represented Ankylos®, Dentsply Friadent implants. A total of 10 disks of each brand were used for the study.

Cell culture and in vitro mineralization: Disks were sterilized and coated with 2% gelatin solution to induce attachment of cells. MC3T3-E1 and C2C12 cells were purchased from ATCC (Manassas, VA, USA). Recombinant human BMP-2 was purchased from GenScript (Piscataway, NJ, USA). MC3T3-E1 and C2C12 cells were cultured in alpha-MEM (Invitrogen, Carlsbad, CA, USA) and DMEM (Invitrogen, Carlsbad, CA, USA), respectively. Culture media were supplemented with 10% FBS (PAA, Etobocoke, Ontario, Canada) and 100 U/ml penicillin–

streptomycin. Cells were grown at 37 °C under 5% CO2 in a humidified incubator. Mineralization of MC3T3-E1 cultures was induced by addition of ascorbic acid (5mg/ml), and phosphate (400mM) to the culture medium for 12 days.

Calcein staining: Cells were fixed with 4% paraformaldehyde. 100 microliters of 0.25% calcein (Sigma-Aldrich, Saint Louis, MO, USA) - 2% $NaHCO_3$ solution prepared in 0.15 M NaCl was added to the cultures and incubated for 5 minutes at room temperature. After washing in PBS, H33258 nuclear staining was performed.

Alamar blue: In order to examine cellular viability/ metabolic activity, Alamar Blue solution (Resazurin sodium salt, Sigma-Aldrich, Saint Louis, MO, USA) was directly added to the medium to 100 μM final concentration. The reduction of Alamar Blue was measured at 560 nm (reference wavelength 610 nm) after 5h incubation at 37 °C using a microplate reader (Infinite 200, Tecan).

Generation of BMP-2 expressing C2C12 cells: C2C12 cells were electroporated together with 0.4 μg of a BMP-2 expression vector (a kind gift from Dr. Katagiri) and 0.1μg of pCMV-Tag, which expresses a neomycin resistance gene. Culture medium was supplemented with 100μg/ml of G418 (Fisher, Pittsburgh, PA, USA) for 9 days. Clones were picked, amplified and screened by alkaline phosphatase (ALPL; a downstream target for BMP-2 signalling) staining (35).

Zymography and Western blotting: Protein samples were prepared in 1 × SDS gel-loading buffer (Laemmli buffer) without adding β-mercaptoethanol and without heating before loading in a 10% SDS-polyacrylamide gel. After electrophoresis, gel was incubated in NBT/BCIP (Roche, Mannheim, Germany) staining solution until the bands corresponding to ALPL were clearly visible. For Western blotting, cells were rinsed with ice-cold PBS and extracted by RIPA lysis buffer (containing 1% NP-40, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 2 mM NaF, 0.5 mM Na_3VO_4, and 1 mM PMSF). Total proteins were quantified using the Pierce BCA protein assay kit (Thermo

Scientific, Rockford, IL, USA). Equal amount of proteins (50 μg) were then subjected to 10% SDS-PAGE and immunoblotting analysis. The primary antibody used for analysis was anti-actin (Sigma-Aldrich, Saint Louis, MO, USA). The secondary antibody was anti-rabbit HRP-IgG (Cell Signaling Technology, Beverly, MA, USA).

Cell proliferation: Nuclear staining was done by H33258 (Sigma-Aldrich, Saint Louis, MO, USA). After washing in PBS, cells grown on the implants were imaged using an inverted fluorescent microscope (Evos FL. Life Technologies) and cell nuclei were counted.

Alkaline phosphatase Immunostaining: BMP2-transfected C2C12 cells were fixed in 4% PFA for 15 minutes, and then blocked with 5% bovine serum albumin (Fisher, Pittsburgh, PA, USA) in TBS-Triton for 30 min at room temperature, followed by overnight incubation with anti- mouse Alkaline phosphatase antibody (R&D systems, Minneapolis, MN, USA). Detection was done by Dylight 488 rabbit anti-goat secondary antibody (Jackson Immuno Research, West Grove, PA, USA) with 1 hour incubation at room temperature. Fluorescence imaging was performed using an inverted microscope (Evos FL. Life Technologies).

Scanning Electron Microscopy (SEM): For SEM, cleaned and sterilized disks in self sealed pouches were received as such from the respective manufacturers. The disks were carefully mounted on stubs, sputter-coated and viewed with Carl Zeiss AG-EVO® 40 series scanning electron microscope.

Statistical analysis: Statistical significance of the differences between the groups was determined using student's *t* test. The statistical power was calculated using the Biomath online software (http://www.biomath.info/power/index.html). We analyzed 10 samples for each group (alpha error 0.05), which corresponds to a statistical power of 92%.

Blinding of the investigators: While performing the experiments, JM (first co-author) was not aware of the sources/manufacturers of the disks, which were identified by their size (small and large). At the end of the analyses, each disk's manufacturer was revealed to her by JSD (first co-author).

Results

Ring culture technique: The variable sizes of the implant disks obtained from two different manufactures demanded an innovative culture system to ensure equal cell density. We achieved this by attaching constant diameter (5mm) plastic cylinders to the disk surface. Disks were sterilized with absolute alcohol and polystyrene cloning cylinders (Sigma) were attached onto the disks using vacuum grease. The disks were then coated with sterile 2% gelatin solution (**Figure 1**).

Increased surface roughness in the 3M™ESPE™ MDIs: Scanning electron microscopy was used in SE mode under 10 kV acceleration voltage for producing the images to observe the surface topography and it showed increased surface roughness in the 3M™ESPE™ MDIs as compared with Ankylos® (**Figure 2**).

Increased proliferation of C2C12 cells grown on 3M™ESPE™ MDI disks: We first examined the proliferation of C2C12 cells treated with BMP-2, a pro-osteogenic cytokine, or without BMP-2 treatment, on both types of disks. 10,000 C2C12 cells were plated and on the following day, the medium was supplemented with 300ng/ml of BMP-2. Cells were grown for 3 days, stained with the nuclear stain H33258 and imaged using fluorescence microscopy. Counting of cell nuclei revealed an increased cell proliferation in C2C12 cells grown on 3M™ESPE™ MDI disks under both conditions, when treated with BMP-2 or without any treatment (**Figure 3**).

Disk type does not affect osteogenic differentiation: C2C12 myoblastic cells were transfected with BMP-2. These cells express high levels of ALPL when compared with the control [untransfected] group (**Figure 4A**). ALPL zymography showed a more intense band indicating very high expression of functional ALPL protein in the stably transfected cells (**Figure 4B)**. The transfected cells were then seeded onto each type of disks (15,000 cells/disk) and were cultured for 3 days. Immunostaining using a goat anti-mouse ALPL antibody revealed a significantly higher number of ALPL-positive cells on the 3M™ESPE™ MDI disks in comparison to Ankylos® disks. Interestingly, when the number of ALPL positive cells was normalized to total cell number, no differences were observed. This finding suggests that the increase of ALPL positive cells was not due to increased cell differentiation, but because of an increase in cell proliferation (**Figure 4.C**).

Increased proliferation of MC3T3-E1 cells and extracellular matrix mineralization on 3M™ESPE™ MDI disks: Pre-osteoblastic MC3T3-E1 cells were plated on each implant disk (40.000 cells per disk) and were differentiated with mineralization medium for 12 days. Quantification of cells after nuclear staining by H33258 revealed an increased number of cells on the 3M™ESPE™ MDI disks (**Figure 5.A)**. Measurement of cell viability by the reduction of Alamar blue® after 3 days of culture of MC3T3-E1 cells further supported an increase of cell proliferation on the 3M™ESPE™ MDI disks. Relative optical density values obtained from the analyses of the respective culture medium were normalized to cell count (**Figure 5.B**).

In order to assess the ability of the system to promote extracellular matrix (ECM) mineralization, MC3T3-E1 cells were plated at equal densities on each disk type and were grown in the presence of differentiation medium for 12 days. Calcein (binds to calcium salts) staining demonstrated an increased mineral deposition on the surface of the 3M™ESPE™ MDI disks when compared with

the Ankylos® disks. Increased cell proliferation in the 3M™ESPE™ MDI disks cultures may explain the increase in ECM mineralization (**Figure 5.C**).

Discussion

In the current study, we used an *in vitro* cell culture system to evaluate the biocompatibility of two implant materials with different surface topography. Our objective was to establish the osseointegration potential of MDIs versus an established regular implant. Disks prepared from the implant material were coated with gelatin to grow cells and proliferation and osteogenic differentiation parameters were evaluated. Considering that the disks obtained from two different sources varied in diameter, we attached 5mm silicon rings to the surface of both types of disks in order to standardize the culture area. The use of vacuum grease created a leak-proof culture well that enabled us to grow and treat cells for the required period of time. Also, it was possible to use limited number of disks as the system was easy to clean, disinfect and reuse.

As cells were grown on metallic surfaces, it was not possible to detect them using light microscopy. This is why we used florescence microscopy to examine the cells and their functional properties once the experiment was complete. Considering that we were unable to routinely examine the live cells on disks during the culture period, we grew same number of cells under identical conditions on a plastic cell culture dish enclosed by the same type of culture rings. These cells were evaluated daily using an inverted light microscope and based on the cell density and the amount of mineral precipitation in this latter culture, we decided to terminate the experiments with the cells grown on the disks.

Two different cell lines were used in our *in vitro* system: C2C12 and MC3T3.E1 cells. Both of these cell lines were developed from mouse tissues. C2C12 cells are myogenic, but retain the potential to express osteogenic markers under appropriate signaling events. Because of their

pluripotency, these cells have been considered as a type of mesenchymal stem cells. It has been shown that when treated with BMPs, these cells readily up regulate many key osteoblast markers including: RUNX2, OSX, osteocalcin and alkaline phosphatase (35). In the current study, we used C2C12 cells that were treated with BMP-2 or stably transfected with a BMP-2 expression vector. MC3T3.E1 cells have been extensively used in numerous cell culture experiments as a model for osteoblasts (36). Under differentiating conditions e.g. in the presence of ascorbic acid and beta-glycerol phosphate these cells up regulate the osteogenic markers and more importantly, promote the deposition of calcium phosphate minerals within and around the collagen-rich extracellular matrix (ECM). In comparison to BMP-2-treated C2C12 cells, MC3T3.E1 cells are considered to be at a more advanced stage of differentiation towards the osteogenic lineage (35).

Our cell culture system was compatible with both cell types as evident by the outcome of various functional studies, which include cell adherence, synthesis of alkaline phosphatase and mineralization of the ECM. However, there was a clear difference in the degree of biocompatibility between the two types of implant surfaces; the 3M™ESPE™ MDI showed higher cell numbers and increased deposition of calcium phosphate minerals in comparison to Ankylos®.

The MDIs are surface treated with sandblasting and passivation with an oxidizing acid (33) whereas Ankylos® implants are sandblasted and acid etched (34). The Scanning Electron Microscopic images showed rougher surface in MDIs in comparison to Ankylos®. The blasting process causes a moderate roughness (1-2 microns) to the implants (33). The surface chemistry and topography of biomaterials seems to play an important role in the success or failure upon placement in a biological environment (37). It has been established that alterations on the surface topography enhances the bone implant contact and biomechanical interaction of the interface during early implantation periods (37).

MacDonald et al have shown that hydrophilic surfaces support cell interactions and biological fluids better than the hydrophobic surfaces (38). It has also been shown that roughening the titanium surface improves hydrophilicity (38). In addition, many authors have stated that rougher surfaces promote differentiation, growth and attachment of bone cells, higher production of growth factors and augment mineralization (39-43). However, an *in vitro* study has demonstrated that osteoblastic cells attach, spread and proliferate faster on smooth surfaces than rough surfaces (44). Alkaline phosphatase is a late osteogenic marker, which is essential for normal bone mineralization. Alkaline phosphatase-deficient osteoblasts fail to mineralize in culture. Considering that there was no significant difference in the relative alkaline phosphatase activity in cells grown on two surfaces, it is unlikely that the surface property of the disks affected cell differentiation. This observation does not support the findings of Davies that BMPs, alkaline phosphatase and osteocalcin, the important markers of osteogenic differentiation and bone tissue formation, express at higher levels on rougher surfaces (45). In addition to surface topography, surface chemistry is also a very strong variable (46, 47). Therefore, the different surface chemistry of the implant materials used by Davies and our group might have contributed to this discrepancy. Regardless, there is a general agreement that roughening the implant surface greater than the degree seen by machining only leads to a stronger bone formation as shown in a systematic review (48).

Our data suggest that the increased cell number is the primary reason why cultures grown on 3M™ESPE™ MDI deposited more minerals in comparison to that grown on Ankylos®. Taken together, we reject the null hypothesis, since our data demonstrates that the MDIs have a superior surface quality that promotes cell proliferation; facilitating osseointegration. However, this needs to be tested *in vivo*.

Conclusions: Our results demonstrate that both implant surfaces are conducive for osteoblastic cell attachment, proliferation and mineralization. However, 3M™ESPE™ MDI surface shows more pronounced effects on cell proliferation, which may in turn facilitate better osseointegration by enhancing ECM mineralization. Our ongoing research will provide further information on how implant surfaces may affect cell behavior including osteogenic differentiation.

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Competing interests:

Jagjit Singh Dhaliwal, Juliana Marulanda, Jingjing Li, Sharifa Alebrahim, Jocelyne S. Feine and Monzur Murshed declare that they have no competing interests in the manuscript. The funding was received from 3M ESPE for a larger study to establish the osseointegration potential of the 3M™ESPE™ MDI for building a scientific evidence. Authors had no commercial interest involved. The comparator implant disks were obtained from Dentsply Friadent for research and comparison purpose only.

Authors' contributions:

JM carried out the cell cultures experiments, analyzed the data and drafted the manuscript. JSD conceived the study and drafted the manuscript. SA established the in vitro culture system. J.Li

generated and characterized the BMP-2-transfected cell line, JSF participated in designing the study. MM provided lab support, designed and coordinated the study, analyzed the data and drafted the final version of the manuscript. All authors read and approved the final manuscript.

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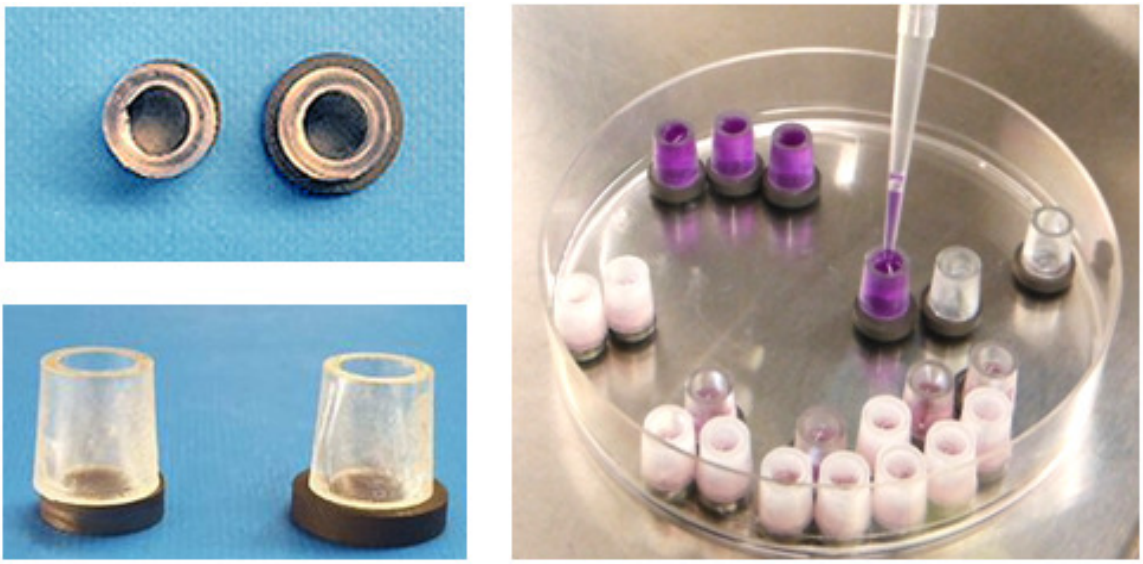

Figure 1: Preparation of specimens. Small discs represent 3M™ESPE™ MDI implants and large disc represent Ankylos® implants. Note that the area of culture remains constant regardless of the disc size.

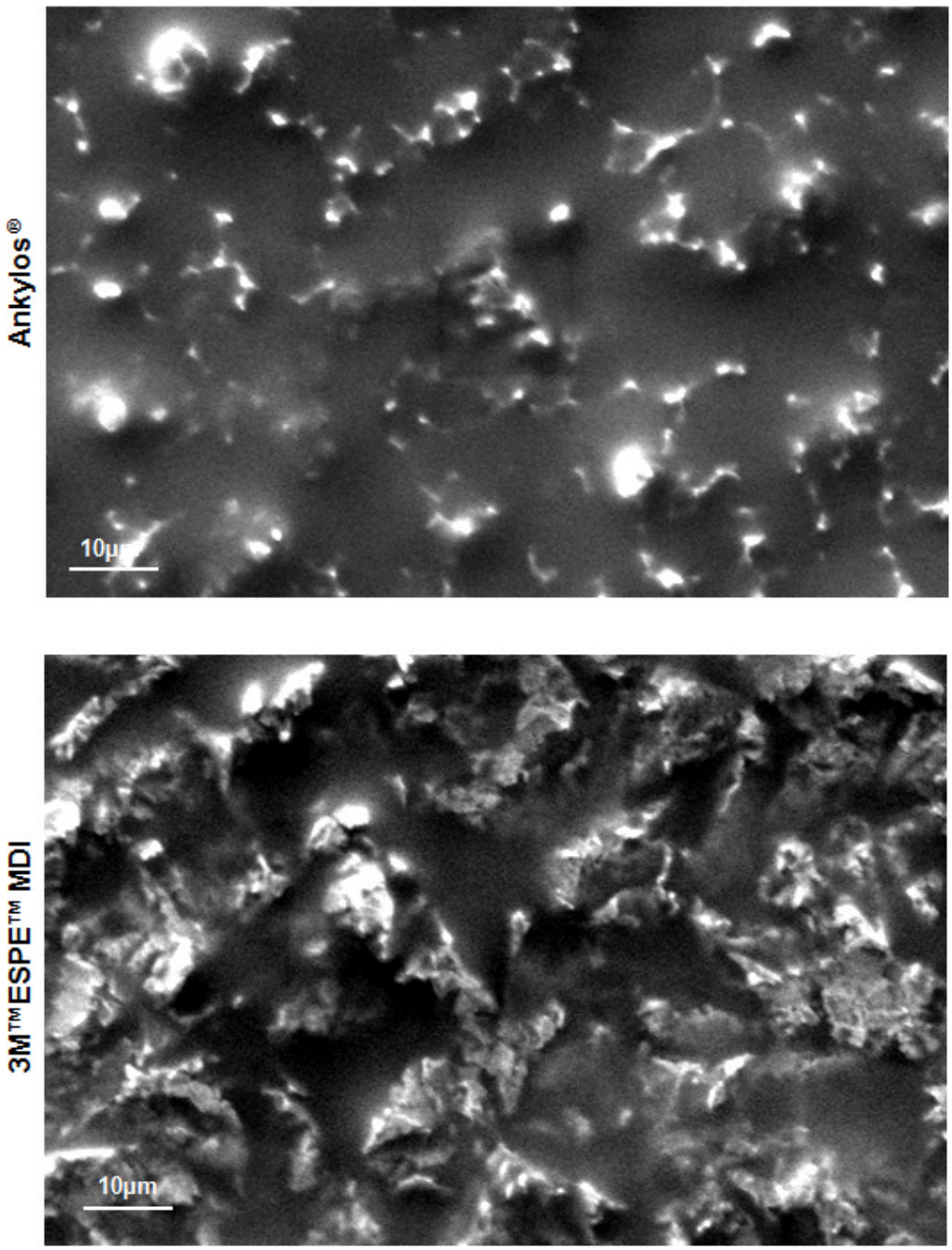

Figure 2: Implant surface characterization under SEM. Increased surface roughness in the 3M™ESPE™ MDI dental implants when compared to Ankylos® implants.

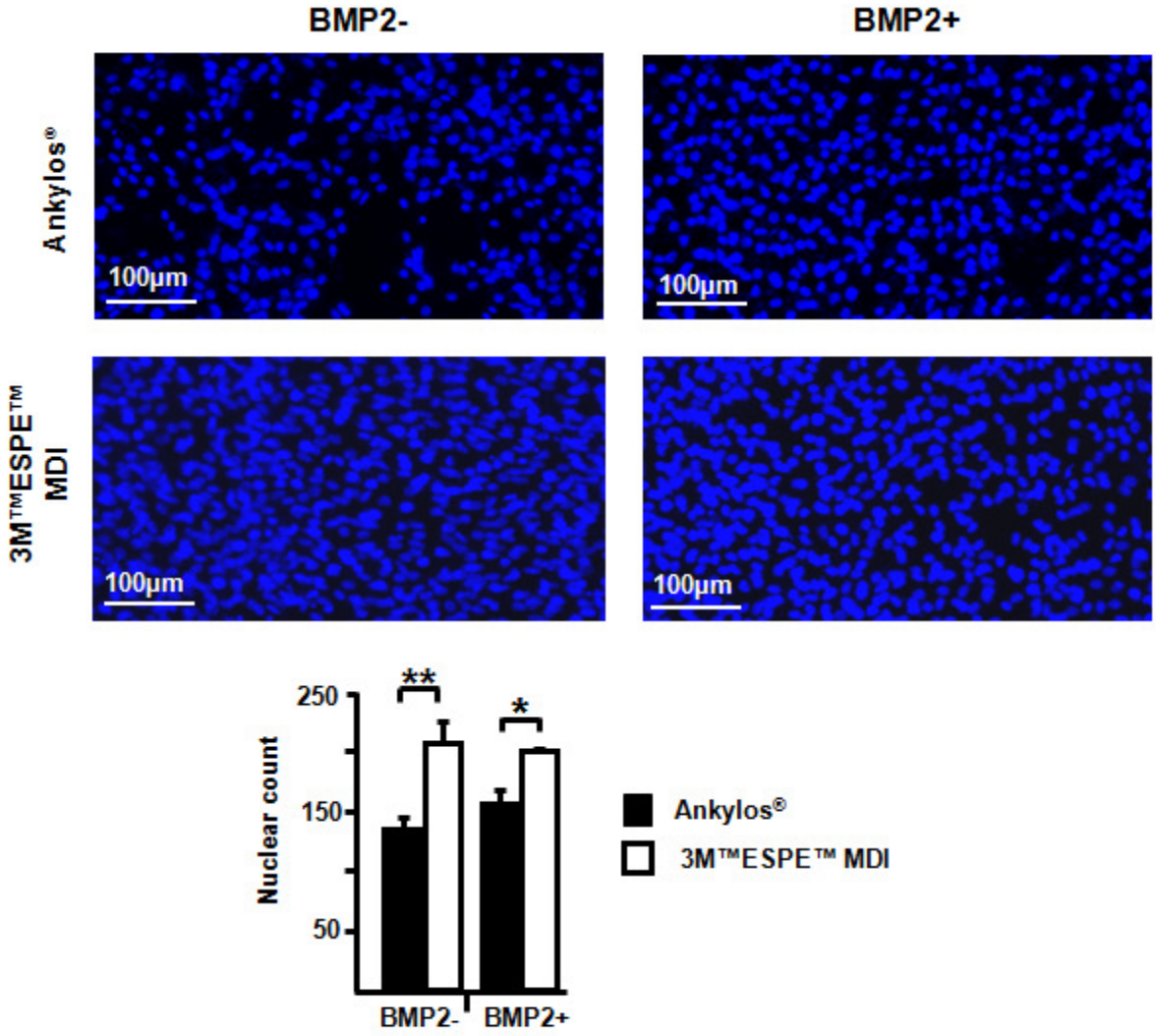

Figure 3: Increased cell proliferation in C2C12 myoblasts grown on 3M™ESPE™ MDI discs in comparison to the cells grown on the Ankylos® discs untreated and treated with bone morphogenetic protein -2 (BMP2).

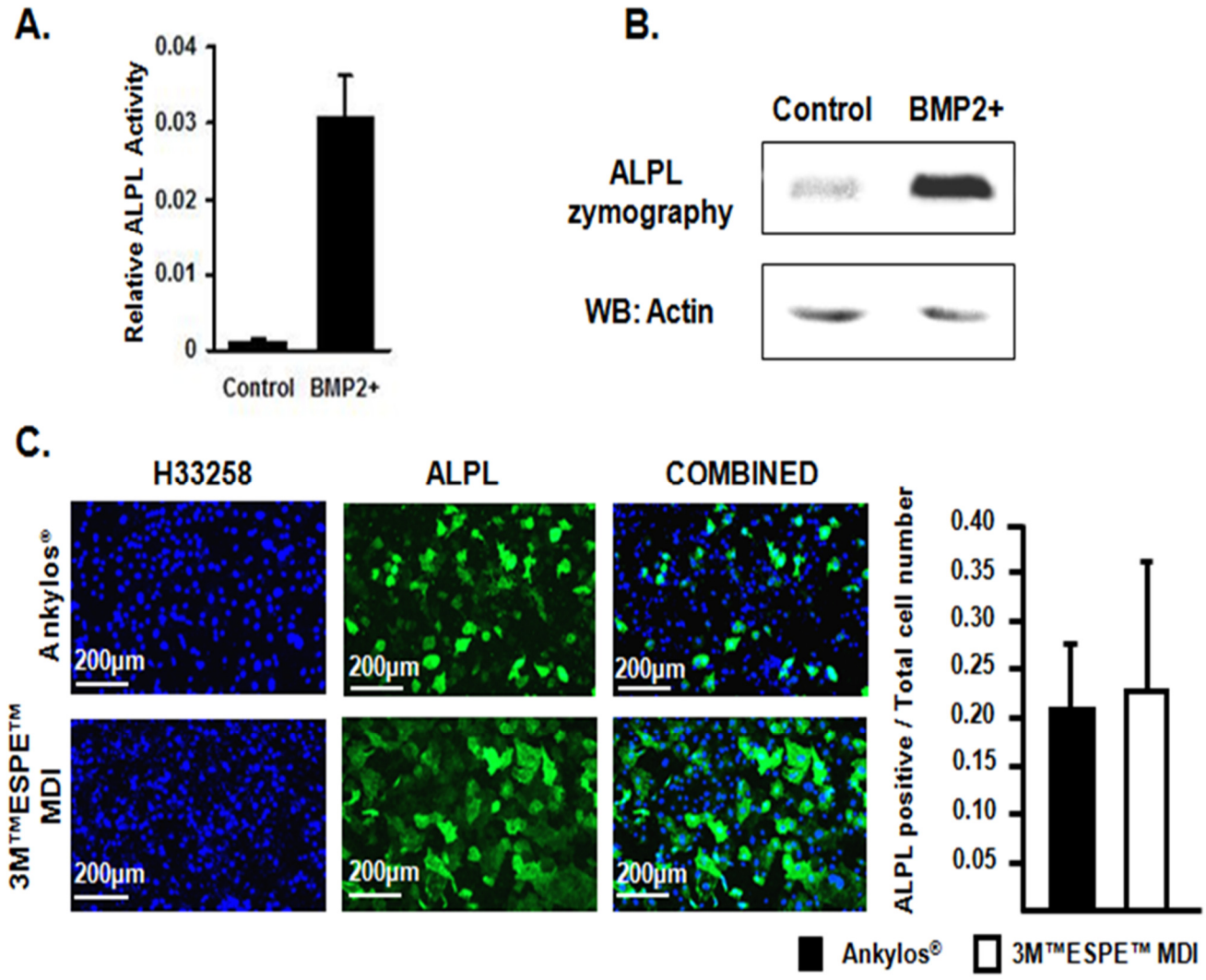

Fig. 4 **A**. C2C12 cells and pBMP2-transfected C2C12 cells were seeded in 24-well plate (50,000 cell/well) and cultured in DMEM medium for 48 h. ALPL assay showing ALPL activity in the BMP2-transfected C2C12 cells. **B**. Cell extracts of C2C12 cells and pBMP2-transfected cells were applied in a natural 10% SDS-PAGE. The gel was then stained with NBT/BCIP solution. Western blotting of actin showing the equal protein loading in the gel (lower panel). C. Increased cell proliferation of C2C12 cells transfected with BMP2 as well as ALPL activity when seeded on 3M™ESPE™ MDI disks. However, when the number of ALPL-positive cells is normalized to the total cell number, no differences are observed.

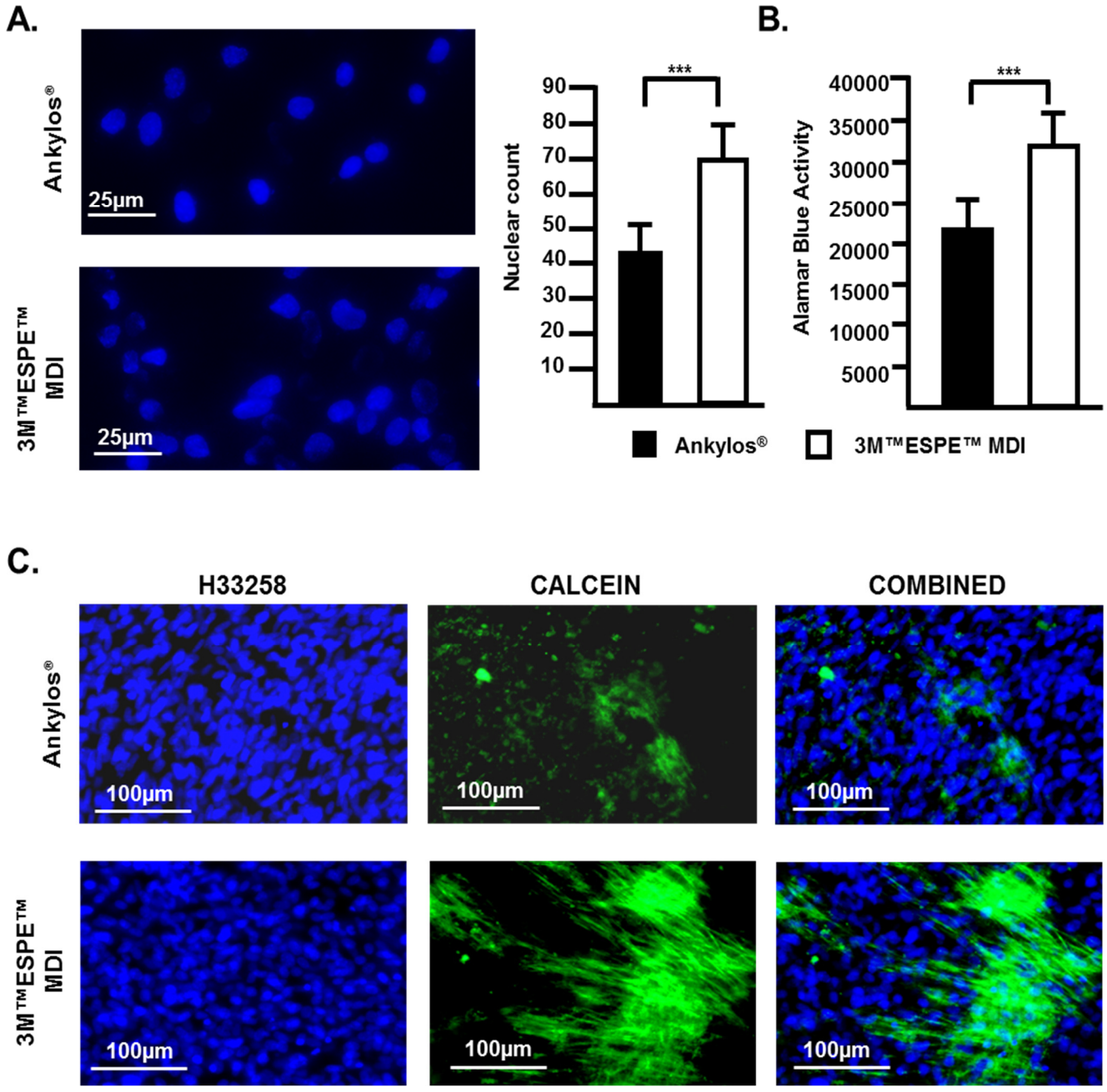

Fig. 5 A. Florescence microscopy showing H33258-stained MC3T3-E1 cells on Ankylos® and 3M™ESPE™ MDI disks. Although equal numbers of cells were plated, after 12 days of culture, more cells were detected on the 3M™ESPE™ MDI disks. B. Increased Alamar blue® reduction in MC3T3-E1 cells seeded on 3M™ESPE™ MDI disks when compared to cells cultured on Ankylos®. C. Increased mineral deposition in the MC3T3-E1 cultures on the 3M™ESPE™ MDI disks in comparison to those on the Ankylos® disks detected by calcein staining.

Chapter four: *In vivo* Study

4.1 Part I

Measuring and Comparing the Stability of Mini Dental Implants and Standard Implants by Resonance Frequency Analysis

Results of the previous *in vitro* study have shown a good response of the osteoblastic cells attachment on the MDI surface. In the following study, we decided to design an experiment *in vivo* in a rabbit model for the stability testing of the MDI and compared it with a standard implant (Ankylos®), the surface of which was also used, for consistency, in the previous *in vitro* experiment.

Stability of implant has a crucial role in achieving and maintaining osseointegration, which is a direct structural and functional contact between the surface of an implant and the surrounding bone. Primary stability is achieved by mechanical union of implant with cortical bone. Thus, it is imperative to measure and quantify initial or primary stability of implants with an easy, non-invasive and predictable test for assessing the long term success. The factors influencing implant stability are quality and quantity of bone where the implant is placed, surgical procedure, diameter, length, shape of the implant (137).

A stable fixation between implant and bone makes it possible for early or immediate loading of implants. The MDIs are usually immediately loaded; therefore, in these demanding situations, it is essential to achieve primary stability. Stability can be measured with various methods like Dental Mobility Checker (DMC), Periotest and Resonance Frequency Analysis (RFA) (95).

Resonance Frequency Analysis (RFA) is a non-invasive method for measuring implant stability using Osstell ISQ equipment (Integration Diagnostics AB, Göteborg, Sweden). The Osstell ISQ device uses magnetic technology for evaluating the stability of implant. Osstell® developed a measurement unit, in lieu of Hertz, for a value in numbers from 1-100 that is called the Implant

Stability Quotient (ISQ). Values ranging from 3,500 to 8,500 Hertz are converted into an ISQ of 0 to 100. This device includes a transducer, which is a metallic rod with a magnet at the end (SmartPeg) (95). The SmartPeg is specific to an implant company, supplied by Osstell® and can be screwed into the inner threads of the implant/abutment. The probe of the device is lightly held on the end of the SmartPeg perpendicular to the alveolar crest. The magnet on the SmartPeg is excited by a magnetic pulse with the probe and the SmartPeg vibrates. The magnet produces an electric voltage in the probe coil which is a signal taken up by the resonance frequency analyzer. However, the MDI is a single piece implant that does not need a separate abutment and has no internal threads. The company does not provide a SmartPeg for these implants. It is important to test the stability of these implants, as they are usually immediately loaded and an RFA measurement is not possible with an Osstell ISQ device.

Our team developed a custom made SmartPeg and tested it in a rabbit model. This was compared with the resonance frequency of MDIs and standard implants. The following manuscript, published in International Journal of Implant Dentistry, is reproduced here.

4.2 Manuscript II

Customized SmartPeg for Measurement of Resonance Frequency of Mini Dental Implants

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Abstract

Background: One-piece narrow diameter implants (NDIs) have been recommended as "Single-tooth replacements in the anterior zones, single posterior, multiple-unit fixed dental prosthesis (FDP), edentulous jaws to be rehabilitated with FDP, and edentulous jaws rehabilitation with overdentures in situations with reduced mesiodistal space or reduced ridge width." (ITI consensus 2013). Since NDIs can be immediately loaded, it is important to be able to carry out stability testing. We developed and validated a customized SmartPeg for this type of implant to measure the Implant Stability Quotient (ISQ). The ISQ of mini dental implants (MDIs) was measured and compared with the stability of standard and in a rabbit model.

Objective: The aim of the study is to test the feasibility of a customized SmartPeg for resonance frequency measurement of single-piece mini dental implants and to compare primary stability of a standard and the mini dental implant (3M™ESPE™ MDI) in a rabbit model after 6 weeks of healing.

Methods: Eight New Zealand white rabbits were used for the study. The protocol was approved by the McGill University Animal Ethics Review Board. Sixteen 3M™ESPE™ MDI and equal number of standard implants (Ankylos® Friadent, Dentsply) were inserted into tibia/femur of the rabbits and compared. Each rabbit quasi-randomly received two 3M™ESPE™ MDI and two Ankylos® implants in each leg. ISQ values were measured with the help of an Osstell ISQ device using custom-made SmartPegs for the MDIs and implant-specific SmartPegs™ (Osstell) for the Ankylos®. Measurements were obtained both immediately following implant placement surgery and after a 6-week healing period. Each reading was taken thrice, and their average compared using Wilcoxon matched pairs signed-rank tests.

Results: The median ISQ and interquartile range (IQR) values were 53.3 (8.3) at insertion and 60.5 (5.5) at 6 weeks for the 3M™ESPE™ MDI and, respectively, 58.5 (4.75) and 65.5 (9.3) for the Ankylos® implant. These values also indicate that both types of implants achieved primary and secondary stability, and this is supported by histological data. ISQ values of both 3M™ESPE™ MDI and Ankylos® increased significantly from the time of insertion to 6-weeks post-insertion ($p<0.05$).

Conclusions: The new custom-made SmartPeg is suitable for measuring the Implant Stability Quotient of 3M™ESPE™ MDIs. The primary stability of 3M™ESPE™ MDIs is similar to the primary stability attained by standard implants in the rabbit tibia.

Keywords: Mini Dental Implants, SmartPeg, Resonance Frequency

Background

Osseointegration refers to the phenomenon for close apposition of the bone to the surface of an implant with no interposing tissue that can be clinically demonstrated by absence of mobility (1, 2). Obtaining primary stability seems to be a precondition for a successful osseointegration (3). Dental implants have a success rate of over 90% and are available in various sizes with different surfaces (4, 5). The diameter of dental implants usually ranges from 3 mm (narrow diameter) to 7 mm (wide diameter), with the majority falling in the "standard diameter" range of 3.7 to 4.0 mm.

Single-piece mini dental implants (MDIs) or narrow diameter implants (NDIs) are being widely used for stabilizing complete dentures (6), orthodontic anchorage (7, 8), single tooth replacements, fixing surgical guides for definitive implant placement, and as transitional implants for support of interim removable prosthesis during the healing phase of final fixtures (9-11).

Due to the MDIs' narrower diameter (1.8-2.4 mm) as compared with regular implants, the width of bone required for their placement is smaller, making the surgery minimally invasive as compared with the surgery for conventional implant insertion (12). In addition, transmucosal placement is performed using a single pilot drill, reducing the need for sutures and long recovery periods (13). Mini dental implants can also be immediately loaded and are cost-effective, which makes them an advantageous alternative for mandibular implant overdentures (13, 14). The success of these implants will depend, however, on their capacity to outstand functional loadings.

Osseointegrated implants are clinically characterized by the absence of mobility, which can be assessed by measuring the primary and secondary implant stability (15, 16). Some authors have suggested that primary stability is a critical factor in predicting whether an implant will be successful or not, and it is considered of highest importance in the long-term success of dental

implants (17, 18). It has also been reported that micro movements can be detected at an early stage by measuring the primary implant stability and that they are unfavorable to the osseointegration of dental implants (19-21).

Mechanical testing methods like reverse torque, or “pullout test”, have been used to study and measure the mechanical interface between implant and bone in various ways (22, 23). The Brånemark group has evaluated the mechanical properties of osseointegrated implants using torsion and pullout tests and lateral loading tests (24, 25). Presence or absence of mobility and the bone level around the implant can be estimated by non-invasive methods based on resonance frequency analysis (RFA) such as those used by Periotest and Osstell™ devices (26-30).

Resonance frequency analysis has been used to document changes in the bone healing along the implant-bone interface by measuring the stiffness of implant in the bone tissue (31-34). It has also been used to determine whether implants are ready for the final restoration (35) or ready to be loaded (33) and to identify the implants at "risk" (36). The first studies using RFA were published in 1996 (37). In 1997, Meredith et al. suggested a non-invasive method for determining the resonance frequency associated with dental implants by connecting an adapter/transducer onto the abutment in an animal study (38). The experimented RFA system, based on magnetic pulses, has been commercially produced as Osstell since the year 2000 (19) (Osstell AB, Göteborg, Sweden). Osstell was later followed by Osstell Mentor™ and Osstell ISQ™. It calculates the Implant Stability Quotient (ISQ) converting kilohertz units to ISQ on a scale of 1-100, where 100 signifies the highest implant stability. Increases in ISQ measurements indicate improved bone stiffness and healing around the implant and better implant stability. The Osstell ISQ works by introducing a controlled vibration to the implant by means of a sensor and a rod (SmartPeg) connected to the implant, and measuring its frequency. These SmartPegs are usually fabricated for standard

diameter implants. The osseointegration potential of single-piece mini dental implants (3M™ESPE™ MDIs) has never been assessed by RFA. The immediate post-surgical ISQ assessment of MDIs is particularly relevant due to their smaller size and surface area in comparison to standard implants.

There are no published studies on the ISQ measurement of mini dental implants, as SmartPegs for these implants are not available till date. Since these are one-piece implants and do not have an internal thread for the SmartPeg's attachment, a custom-made SmartPeg needs to be fabricated for ISQ measurement. Therefore, we developed and tested a customized SmartPeg for 3M™ESPE™ MDIs to measure the ISQ.

Objective

The aim of the study is to test the feasibility of a customized SmartPeg for ISQ measurement of single-piece mini dental implants and to compare the primary stability of a standard and the mini dental implant (3M™ESPE™ MDI) in a rabbit model after 6 weeks of healing.

Methods

Development of a customized SmartPeg

Single use Osstell SmartPegs for standard implants are made from a soft metal with a zinc-coated magnet mounted on top of it and attached to the implants or abutments' internal threads. As the company does not provide SmartPegs for one-piece implants, we developed a customized SmartPeg for mini dental implants (3M™ESPE™ MDIs), which do not have internal threads (Figure 1). After confirming that the standard SmartPegs™ are fabricated in aluminium, we customized a prototype in the same metal with a square-shaped assembly, which could be tightened with a small screw over the spherical top end of the MDIs. Our SmartPeg prototype was tested for

reproducibility verifying the ISQ values on an MDI inserted into a wooden plank made of balsa wood. RFA measurements were taken 50 times, and a standard error of mean of all measurements was calculated.

Animal model and sample size

Eight clinically healthy New Zealand white rabbits weighing >3.5 kg used for the study were housed in the Central Animal House facility. The head of tibia/femur of the animals were chosen for the implantation of samples because they have been widely used as an animal model, and so, our results could be promptly compared (39-46). The sample size of this study has been calculated based on the results of a similar study (36). It was expected that 88% statistical power would be achieved by using sixteen 3M™ESPE™ MDIs (experimental) and equal number of regular implants Ankylos®, Dentsply Friadent GmbH (control). Each animal received two implants on each of the hind limbs, right and left tibia/femur head, quasi-randomly. Therefore, each animal received a total of 4 implants (2 experimental and 2 regular implants).

Surgical procedures

The procedures were approved by the institutional animals' ethics review board of McGill University, Montreal, Canada. Adequate measures were taken into consideration to minimize pain and distress in the animal during the procedure. Animals were anaesthetized by intravenous injections of a ketamine hydrochloride-xylazine mixture at 35-50 mg/kg and 1-3mg/kg, respectively, according to the method described by Green et al. (47). Acepromazine was injected subcutaneously at the dosage of 1mg/kg. Further injections of the mixture were given to maintain anesthesia, if necessary. All surgical procedures were performed in accordance with McGill's Standardized Operating Protocol (SOP).

For the MDIs, a small longitudinal skin incision was made just distal to the tibia/femur joint. The tibia/femur head was exposed subperiosteally and an osteotomy was performed with the pilot drill under copius irrigation with saline solution, transposing the cortical bone to the depth of 0.5 mm. The implants were aseptically transferred to the bone site and manually rotated clockwise while exerting downwards pressure to start the self-tapping process. When bony resistance was encountered, the winged thumb wrench was used for driving the implant deeper into the bone, if necessary.

Ankylos® implants were inserted in the other tibia/femur head of the animals according to the manufacturer's protocol as follows: After mobilizing the subperiosteal flap and using a 3-mm center punch to register a guiding point for the osteotomy, a twist drill, depth drill series and a conical reamer were used sequentially to complete the osteotomy and to develop a conical shape for accomodation of the implant's body. A counter clockwise rotation was used to compress the bone in case of soft bone. The tap or thread cutter was used to create the threads in dense bones. Following, the implant assembly was asseptically transferred to the osteotomy site, and the implant placement was started manually and finalized using a hand ratchet. If excessive force was experienced, the osteotomy was irrigated and the depth was checked by retapping.

Resonance frequency assessment

Resonance frequency assessment was performed thrice, just after the insertion of the implants, using the Osstell ISQ™ device. In brief, customized SmartPegs were stabilized onto the head of the 3M™ESPE™ MDIs and Osstell Company's specific SmartPeg™ devices were screwed into Ankylos® implants, taking care to ensure that no significant torquing force was applied to the implants, and the RFA was carried out. These procedures were repeated for post-euthanasia RFA.

Post-surgical treatment and euthanasia

Rabbits were given a dose of Cephalexin 12mg/kg 0.5mL IV once intraoperatively and a postoperative analgesic, Carprofen 2-4mg/kg SC every 8 hours for 3 days, according to McGill's SOP. The animals had a free access to water and food, and routine daily care followed as per McGill's SOP#524.01. The sutures were removed after 7-10 days and the animals were euthanized at 6 weeks postoperatively. It has been shown by various authors that this period is adequate to develop a "rigid osseous interface" in rabbits (30). An overdose of pentobarbital sodium 1ml/kg intravenously was used for this purpose (48).

Statistical analyses

ISQ values were averaged and compared between implant types and times using Wilcoxon's matched pairs signed-rank test*s* at a significance level of $p<0.05$. Statistical analysis was performed with the help of SPSS statistical software version 17.

Results

The ISQ values obtained while calibrating the customized SmartPeg were similar to *in vivo* results. Median ISQ values at insertion and at 6 postoperative weeks were 53.3 (IQR 8.3) and 60.5 (5.5) for the 3M™ESPE™ MDIs, and 58.5 (4.75) and 65.5 (9.3) for the Ankylos® implants, respectively, with no statistical difference (Figures 2 & 3). ISQ values of both 3M™ESPE™ MDI and Ankylos® (Figures 2 and 3) increased significantly from the time of insertion to 6-week post-insertion ($p<0.05$).

Discussion

It is important to measure the Implant Stability Quotient (ISQ) of single-piece mini dental implants as they are becoming increasingly popular, with the concomitant increase in publications

demonstrating their high survival and success rates. Although the clinical use of Osstell devices is also increasing, there is lack of studies on its use with single-piece implants, which do not have internal threads. Implant Stability Quotient (ISQ) is an objective and standardized method for measuring implant stability clinically ranging from 55 to 80, with higher values usually observed in the mandible (49). The ISQ scale has a non-linear correlation to micro mobility. With more than 700 scientific references, we now know that high stability means >70 ISQ, between 60 and 69 is medium stability and < 60 ISQ is considered as low stability.

The rabbit tibias have been used to determine longitudinal changes in the resonance frequency and measured for over 168 days from the time of implant insertion and it was observed that resonance frequency values increased over time (38).

However, the relationship between the bone density and ISQ is not significant (50). Therefore, higher ISQ values are a sign of bone anchorage of implants, but the relationship of resonance frequency analysis with bone structure is unclear (51-53). ISQ values decline in the first 2 weeks after implant insertion, and these changes may be associated with early bone healing and marginal alveolar bone resorption. Bone remodeling reduces primary bone contact. In the early stage after implant placement, the formation of bony callus and increasing lamellar bone in the cortical bone causes major changes in bone density. Therefore, in the healing process, primary bone contact decreases and secondary bone contact increases (53, 32). Degidi et al (54) reported that there may also be a discrepancy as the histological analyses is a two-dimensional picture of the three-dimensional bone-implant contact.

If the initial ISQ value is high, a small drop in stability normally levels out with time. A big drop in stability or decrease should be taken as a warning sign. Lower values are expected to be higher

after the healing period. The opposite could be a sign of an unsuccessful implant, and actions should be taken accordingly.

Studies have shown that the resonance frequency value is greatly associated with the quality and quantity of bone-implant contact (31, 38). There is a positive correlation between resonance frequency analysis and histomorphometric measurements (37). In our histological study previously reported, similar findings were demonstrated (55).

Our results indicate that both types of implants achieved primary and secondary stability.

Several measurements may be more dependable than single measures; therefore, it may be important to measure resonance frequency multiple times and average the values in order to obtain the most reliable assessment. While reliability of resonance frequency analysis has not been established in the past for these mini dental implants used for overdentures, studies have shown similar or lower levels of reliability for regular dental implants (56).

In general, there was an increase in the ISQ values in both groups, which may be related to enhancement of rigidity between the implants and neighboring tissues and largely with the changes at the bone-implant interface. It has been demonstrated that there is a development of woven bone surrounding the implants 1 week following placement in the rabbit tibia. This scantily organized bone is resorbed by osteoclasts and slowly remodeled into lamellar bone and gets more compacted around the implant surface and remodeled to become a mature bone over a period of 42 days (38, 57). There seems to be minimal changes in the resonance frequency after this period. Our results are in concurrence with the study by Meredith et al (38).

As there are no studies that provide data based on resonance frequency measurements for single-piece MDIs, the exact RFA threshold values for MDIs may have to be identified with more studies conducted *in vivo*.

The resonance frequency assessment with a customized SmartPeg would be a useful tool to provide clinically useful information about the condition of the bone-implant interface of 3M™ ESPE™ MDIs. Frequently, implant failures are associated with biomechanical reasons; implant stability assessment can reduce this to a great extent. The higher the RFA value, the higher the success in implant treatment and the lower the risk for failure in the future. On the other hand, lower RFA values may indicate greater risk for implant complications. The MDIs are usually immediately loaded. Resonance frequency measurement technique is also of value in evaluating the immediate loading implants (58). The results of the present study are encouraging and show that it is possible to measure ISQ for these single-piece MDIs. This study is the first of its kind and similar type of studies should be conducted among humans, to make the results more meaningful and generalizable.

Conclusions

The results of this animal study indicate that ISQ measurement of these single-piece MDIs is possible with the help of a custom-made SmartPeg and that 3M™ESPE™ MDIs attain primary and secondary stability at the same levels as standard implants in the rabbit tibia.

Authors' contributions: JSD carried out the experiments and drafted the manuscript, RA conceived the study and helped in revising the manuscript, AF contributed to the designing the SmartPeg, SK helped in the data analysis, JSF participated in this study's design and overall coordination. All authors read and approved the final manuscript.

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Competing interests

Jagjit S. Dhaliwal, Rubens F. Albuquerque Jr., Ali Fakhry, Sukhbir Kaur and Jocelyne S. Feine declare that they have no competing interests.

Ethical Approval: IRB approval, Animal Use Protocol # 2012-7221, was provided by Suzanne Smith, Director of the Animal Compliance, McGill University, Montreal, Canada.

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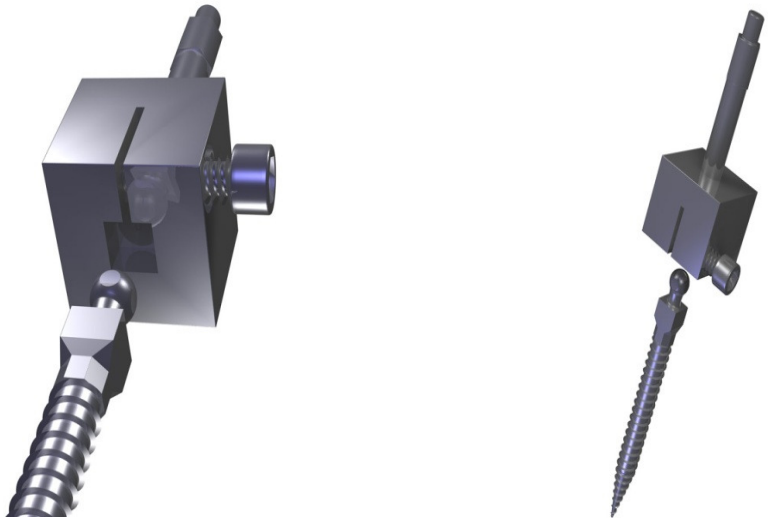

Figure 1. Customized SmartPeg diagrams

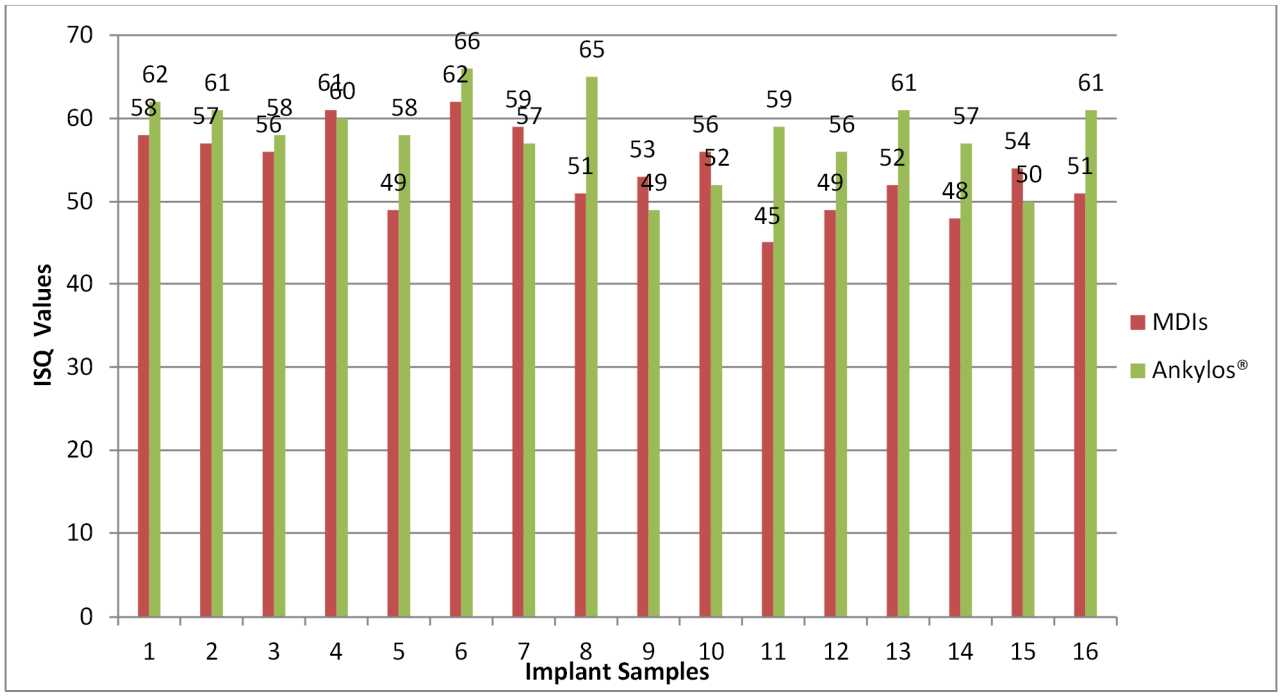

Figure 2. ISQ values of MDIs and Ankylos® immediately upon insertion

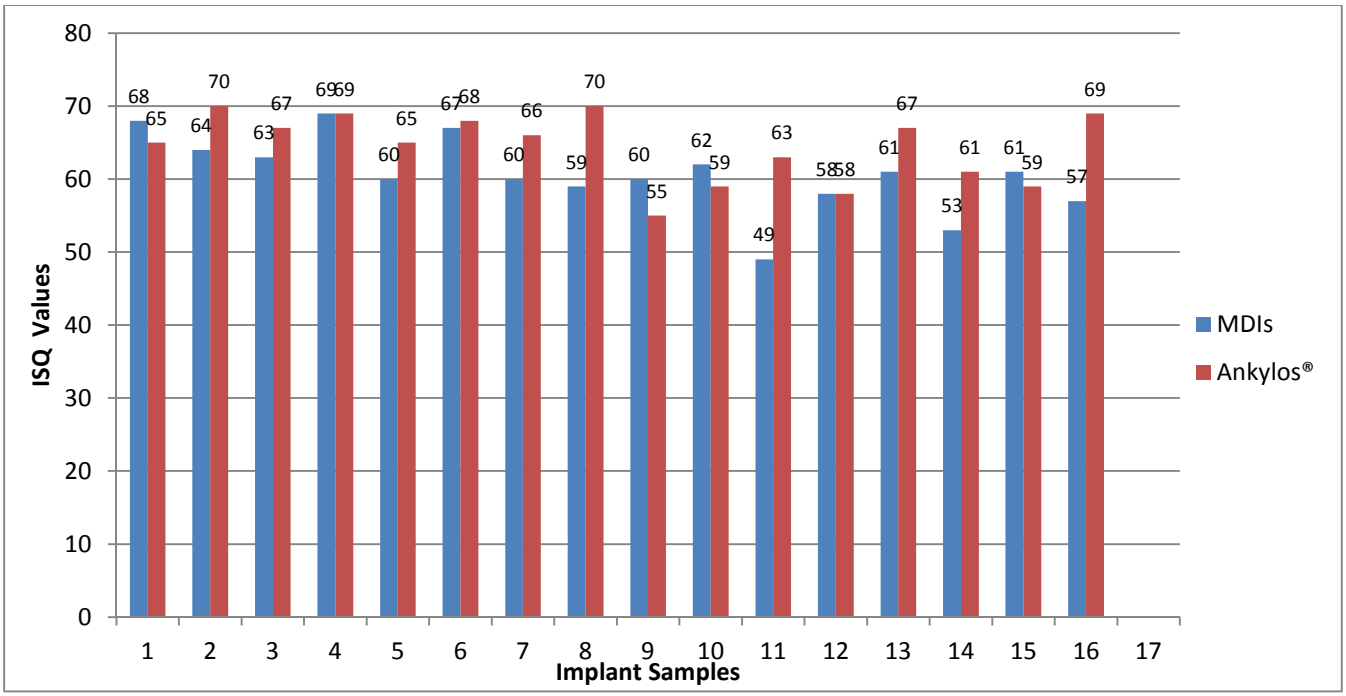

Figure 3. ISQ values of MDIs and Ankylos® after euthanasia

4.3 Part II

Comparing Bone Apposition on the Surface of Mini Dental Implants and Standard Implants with Histomorphometric Methods

Results of the previous *in vivo* study in rabbits have shown that the MDIs and Ankylos® are able to attain similar levels of primary and secondary stability. It is important to preserve primary stability, which is attained by passive mechanical fixation in the host bone immediately after implant placement. The secondary implant stability is influenced by the bone tissue response to surgical procedures and implant surface. Secondary implant stability is also achieved subsequently by bone resorption and newly formed bone which leads to osseointegration. Achieving secondary implant stability with osseointegration is fundamental to a successful implant treatment (138).

Histomorphometric analysis is done by calculating the quantity of the peri-implant bone and bone-implant contact (BIC) from a dyed specimen. A few histological sections per sample are usually used for the estimation of mean values of peri-implant bone formation (139). Accurate measurement of bone around the implant is an advantage, as available bone volume is regarded as a key factor in attaining implant success. Donath and Breuner (139) had developed a method for studying undecalcified sections of bone and teeth which has also become relevant for dental implants. A sawing and grinding method is used to prepare thin bone and implant sections. These are then analyzed by staining at a thickness of 20-30 μm. This method has been the origin for the measurement of Bone Implant Contact (BIC). A high amount of bone contacting a number of implant threads microscopically, demonstrated evidence of an implant embedded in bone (140). Bone Implant Contact can also be done with other methods such as electron microscopy (141). However, the histomorphometric evaluation of the BIC was recognized as the most widely used technique and was employed in most of the investigations. BIC is critical in generating secondary implant stability and higher BIC is generally assumed to result in a better implant stability (142).

In the following study, we decided to design an experiment for the histological evaluation of the Bone Implant Contact area of 3M™ESPE™ MDIs and compared it with a standard implant (Ankylos®). The manuscript has been published in the *International Journal of Implant Dentistry* and is reproduced here.

4.4 Manuscript III

Osseointegration of Standard and Mini Dental Implants: A Histomorphometric Comparison

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Dhaliwal et al. International Journal of Implant Dentistry (2017) 3:15.

Abstract

Background: Mini dental implants (MDIs) are becoming increasingly popular for rehabilitation of edentulous patients due to their several advantages. However, there is a lack of evidence on the osseointegration potential of the MDIs. The objective of the study was to histomorphometrically evaluate and compare bone apposition on the surface of MDIs and standard implants in a rabbit model.

Methods: Nine New Zealand white rabbits were used for the study utilized to meet statistical criteria for adequate power. Total 18 3M™ESPE™ MDIs and 18 standard implants (Ankylos® Friadent, Dentsply) were inserted quasi-randomly into the tibia of rabbits (four implants per rabbit); animals were sacrificed after a 6-week healing period. The specimens were retrieved en bloc and preserved in 10% formaldehyde solution. Specimens were prepared for embedding in a light cure acrylic resin (Technoovit 9100). The most central sagittal histological sections (30-40µm thick) were obtained using a Leica SP 1600 saw microtome. After staining, the Leica DM2000 microscope was used, the images were captured using an Olympus DP72 camera and associated software. Bone implant contact (BIC) was measured using Infinity Analyze software.

Results: All implants were osseointegrated. Histologic measures show mineralized bone matrix in intimate contact with the implant surface in both groups. The median BIC was 58.5 % (IQR 8.0) in the MDI group and 57.0 % (IQR 5.5) in the control group ($P > 0.05$; Mann-Whitney test). There were no statistical differences in osseointegration at 6 weeks between MDIs and standard implants in rabbit tibias.

Conclusion: Based on these results, it is concluded that osseointegration of MDIs is similar to that of standard implants.

Keywords: Bone implant contact, Mini dental implant, Osseointegration

Introduction

The term “osseointegration” was first introduced to explain the phenomenon of stable fixation of titanium to bone by Brånemark et al. in the1960s (1). Osseointegrated implants were introduced, a new era in oral rehabilitation began, and many studies were conducted (2, 3). A success rate of over 90% has been reported (4, 5). Further, a success rate of 81% in the maxillary bone and 91% in the mandible can be accomplished (6). Dental implants have been widely used for the stabilization of complete dentures and also help to maintain bone, function, esthetics, and phonetics and improve the oral health-related quality of life (7). The dental implants are available with different surfaces and sizes. The size of the dental implants usually ranges in the diameter range of 3 mm (narrow diameter) to 7 mm (wide diameter). However, majority of the implants fall in the “standard diameter” range of 3.7 mm to 4.0 mm (8).

Mini dental implants or small implants are also being widely used for stabilizing the complete dentures (9), for orthodontic anchorage (10-12), single tooth replacements (13, 14), fixing the surgical guides for definitive implant placement (15), and as transitional implants for the support of interim removable prosthesis during the healing phase of final fixtures (16, 17).

The single-piece MDIs are becoming increasingly popular for the purpose of denture stabilization. There are many advantages of the MDIs over the regular implants. The surgery is minimally invasive compared with conventional implant surgery, which helps in decreased morbidity for the patient. Transmucosal placement is possible using a single pilot drill, and these can often be loaded immediately (18). Gingival healing is typically seen in 2 to 5 days, extended healing period with MDIs is usually not necessary (19). The insertion of MDIs needs a minimal disturbance of the periosteum, thus osseointegration process is accelerated and time needed for MDIs tends to be considerably small than that of regular implants due to less injurious insertion procedure (9). The

need for sutures or long recovery periods is eliminated (3). The patient can walk in to the office into the office the morning and is out the same day with a full set of teeth, the patient is allowed to eat the same day. These can work well for patients who have significant bone loss that restrict them from being a candidate for regular dental implants. MDIs are also a solution for patients that cannot have surgery for medical reasons. MDIs are also cost effective (20). Considerable confusion exists in the literature regarding the best method to monitor the status of a dental implant. Various methods have been used to demonstrate the osseointegration of dental implants. A common and time-tested method to evaluate biological responses to an implant is to measure the extent of Bone Implant Contact (BIC), referred to as histomorphometry at the light microscopic level. Bone implant contact (BIC) is one of the parameters which has been used extensively to study the amount of bone apposition next to the implants (21-27). When an implant is placed in the jaw, it is in contact with compact bone as well as cancellous bone. The different structures of the two types of bone frequently result in variation of mineralized bone-to-implant contact length along the implant surface (28, 29). Albrektsson et al. identified the key features affecting osseointegration about 4 decades ago, e.g., implant surface and topography, surface chemistry, charge and wettability (30). Roughness and enhanced surface area seem to be helpful for osseointegration. Carlsson et al. reported that screw-shaped implants with a rough surface had a stronger bonding than implants with a polished surface (31). A coarse surface seems to be more appropriate for osseointegration of implants than a relatively smoother implant surface by representing a greater degree of implant integration (32-34). The bone contact areas of 3M™ESPE™ MDIs are surface treated. The treatment process of these MDIs includes sandblasting with aluminium oxide particles followed by cleaning and passivation with an oxidizing acid (35).

Despite the advantages of the mini dental implants, evidence on their efficacy and long-term success is lacking. The success of these implants will depend on their union with the surrounding bone. New implant systems entering into the market have to be studied with the help of animal models first, to demonstrate the osseointegration potential for their probable success in humans. There is a limited evidence regarding the 3M™ESPE™ MDIs. Therefore, there is a need for an animal study to explore the osseointegration of these implants to assist in better understanding of the treatment selection, prognosis, and outcomes for the patients.

Objectives of the study

The objective of this study is to compare bone apposition on the surface of mini dental implants and standard implants by means of histomorphometric methods.

Methods

Animal Model: Nine clinically healthy New Zealand white rabbits weighing 3.5 kg and more were used for the study, and the animals were housed in the central animal house facility. The heads of tibia/femur of the rabbits were used for the implantation of samples. Rabbits' tibiae and femur have been widely used as an animal model by various other authors to study osseointegration of dental implants (36-45).

Sample size: The sample size of this study has been calculated based on the results of a similar study by Bornstein et al. (22). It was established that 88% statistical power will be achieved by using 18 mini dental implants (3M™ESPE™ MDIs) for the experimental implants and equal number of an established regular implant (Ankylos®, Dentsply Friadent GmbH) for the control. Therefore, the total number of implants used was 36. Each animal received four implants on hind

limbs (right and left tibia/femur head) quasi-randomly (the heads of tibia and femur have been chosen to get the maximum bulk of bone). Therefore, each animal received two experimental and two regular implants.

Surgical Procedure: The procedures were approved by the institutional animals' ethics review board of McGill University, Canada. Animals were anaesthetized by an intravenous injection of ketamine hydrochloride-xylazine mixture at 35-50 and 1-3mg/kg respectively according to a method described by Green et al. (46). Acepromazine was injected subcutaneously at dosage of 1mg/kg. Further injections of the mixture were given to maintain anesthesia, if necessary (46). Sterile ophthalmic ointment was put in both eyes to prevent corneal desiccation. Animals were shaved for twice the size of the expected surgical field with an electric razor. All loose hair and debris from the animal were removed. The surgical area was cleaned with gauze and 2% chlorhexidine solution to remove the majority of debris from the surgical site. Antiseptic skin preparation was done starting at the center of the surgical site and moved to the outside of the prepared area in a circular manner. Three scrubs with 2% chlorhexidine solution and three alternating rinses with alcohol were performed. The animal was draped and fixed with clamps on a sterile, impermeable covering to isolate the disinfected area. This was performed by the gloved and gowned surgical team under sterile conditions.

Surgical protocol for 3M™ESPE™ MDIs: A small longitudinal skin incision just distal to the tibia-femur joint was made. The tibia/femur head was exposed subperiosteally and an osteotomy performed with the delicately placed pilot drill over the entry point and lightly pumped up and down under copius saline irrigation just to enter the cortical bone for the MDIs. This was used for initial bone drilling to a depth of 0.5 mm. The 3M™ESPE™ MDIs (size 1.8 mm X 10 mm) vial was opened and the body of the implant was firmly grasped with a sterilized locking pliers. The

titanium finger driver was attached to the head of the implant. The implant was transferred to the site and rotated clockwise while exerting downwards pressure. This began the self-tapping process and was used until noticeable bony resistance encountered when it touched the lower cortical plate. The winged thumb wrench was used for driving the implant deeper into the bone, if necessary. All the animals received one MDI on the head of each tibia or femur. Therefore, total18 mini dental implants were inserted.

Surgical protocol for the Ankylos® implants: An equal number of comparator implants (size 3.5 mm X 8 mm) were inserted in the other tibia/femur heads of the animals after performing the osteotomy according to the manufacturer's protocol as follows. After mobilizing the mucoperiosteal flap, the 3-mm center punch was used to register a guide for the twist drill. The twist drill was used to establish the axial alignment of the implant and to assist in the guidance of the depth drill. The depth drills were sequentially used to create osteotomy to the subcrestal axial depth of 0.5 mm. The conical reamer was then used to develop the conical shape of the implant body and to check the osteotomy depth. A counter-clockwise rotation was used to compress the bone in soft bone. The tap or thread cutter was used for dense bone to create the threads in the osteotomy, with the thread cutter's diameter corresponds to the implant diameter. To engage the implant into the implant placement tool, the square faces on the implant fixture mount were aligned with those on the implant placement tool, then pushed together. Using the handle (finger wheel), the implant was pulled out of the inner vial and the plastic collar was discarded. The implant placement assembly was transferred to the osteotomy and the implant was secured into the osteotomy site. The implant placement was started with the handle and finally placed using the hand-ratchet. If excessive force was experienced, the osteotomy was rinsed out and the depth was checked by retapping. To disengage fixture mount from implant, the open-ended spanner was used

to break the retention force of the fixture mount retention screw. The knurled top of the implant placement tool was turned by hand to fully disengage the fixture mount from the implant. Pushing down on the knurled top of the implant placement tool disengaged the fixture mount.

Suturing: Expected length of the procedure was approximately 1 hour. Following placement of the implants, the wound was sutured in layers. The underlying muscle, fascia and dermal layers were sutured with the help of a Vicryl (Polyglactin 910) suture with 3/8 circle reverse cutting needle. The skin was sutured to a primary closer with the same suture material.

Radiograph: Plain X-ray images of all the rabbit tibia were taken after suturing to confirm the position of implants and to detect any injury/fracture of the bone (Fig. 1).

Post-Surgical treatment: After the surgical procedure, the animals were housed in a cage under the supervision of a veterinary doctor until they came out of anaesthesia. The rabbit was observed every 2 hours on the first day of surgery followed by once a day to check the wound for infection. The wound was protected with povidone iodine ointment. The rabbits were allowed immediate weight bearing as tolerated; therefore, they had no restraints on weight bearing.

The animals were shifted and housed together with other rabbits. The rabbit was given a dose of Cephalexin 12mg/kg 0.5ml I.V. once intraoperatively and a post-operative analgesic, (Carprofen 2-4mg/Kg) S.C. every 8 hourly for three days according to McGill SOP. The routine daily care was as per McGill SOP#524.01.

The feeding protocols were followed according to the university central animal house facility guidelines. The animals had free access to water and feed. The sutures were removed after 7-10 days, and the wound was cleaned with 0.2% chlorhexidine solution.

Euthanasia: The animals were euthanized at 6 weeks respectively. An overdose of pentobarbital sodium 1ml/kg intravenously, under general anaesthesia, was used for this purpose (47, 48).

Specimen retrieval: The implants along with their surrounding bone were excised with a surgical saw immediately following the euthanasia. The excess tissue was dissected and the specimens were removed *en bloc* with a margin of surrounding bone of about 5-10 mm. The specimens were immediately placed into the 10% formaldehyde solution.

Sample preparation for embedding: The specimens were dehydrated in the ascending graded ethanol solution and kept in a pre-filtration solution for 3 hours at room temperature and then in the filtration solution at 4^0C for 17 hours. The specimens were then embedded in a light curing resin Technovit 9100 NEW (Kulzer & Co., Wehrheim, Germany) polymerization system based on methyl methacrylate, specially developed for embedding mineralized tissues for light microscopy. The polymerization mixture was produced by mixing the solution A and B in the proportion of 9 parts A and 1 part of solution B directly before use. This was done in a beaker by using a glass rod to stir the mixture. The samples were then positioned in the labeled plastic moulds, completely covered in the polymerization mixture, and placed in cooled desiccators and under a partial vacuum at 4^0C for 10 min. The resulting blocks were placed in a sealed container and left to polymerize between -8^0C and -20^0C. The samples were allowed to stand at 4-8^0C in the refrigerator for at least 1 hour before allowing it to slowly come to room temperature. The polymerization times are dependent on the volumes of polymerization mixture used and of the constancy of the temperature at which polymerization is carried out.

Preparation of histological sections: The acrylic block was mounted into the object holder of the Leica SP 1600 saw microtome (Figure 2). The height of the object was adjusted until the surface

of the object is slightly above the upper edge of the saw blade. The surface of the block was trimmed to get a plane surface prior to producing slices of a defined thickness. During the sawing process, the water flow was adjusted so that the water jet lands on the edge of the saw blade. The built-in water cooling device prevents overheating of the object and removes saw dust from the cutting edge and thus prolongs the lift time of the saw blade. The most favorable feed rate was determined (Figure 3). After trimming, the first undefined slice was removed from the saw blade. The desired section thickness was selected, considering the thickness of the saw blade and added to the desired thickness of final section. The section was stabilized during the sawing process. To do so, a glass cover slip was glued onto the trimmed surface of the specimen block using cyanoacrylate glue. These blocks were cut with a low speed saw under water along the lateral surface of the implant (47, 48). The implant bearing blocks were cut parallel to the long axis of the implant, and 30-µm-thick specimens were obtained.

The saw blade has a thickness of 280 µm and a feed of 310 µm was selected to obtain the final section thickness of 30 µm. The knurled screw was used for the setting of the section thickness. The prepared section was finally removed from the saw blade. The specimens were prepared for histology by the method as described by Donath and Breuner (49).

Histological evaluation: Subsequently, the sections were stained with methylene blue and basic fuchsin similar to other studies (21, 22, 50). The specimen sections were evaluated at the most central saggital section of each implant under optical microscope after staining. The images were photographed with a high resolution camera and interfaced to a monitor and PC, observed under the Leica DM2000 microscope, and the images were captured using an Olympus DP72 camera and associated software (4, 21, 22). Bone implant contact (BIC) was measured using Infinity Analyze software. Six images of the same implant were taken and measurements were done. The

percentage of the interface contact length between implant surface and bone, bone implant contact (BIC), was calculated. The percentage of bone tissue in 200-µm-wide zone parallel to the contour of the implant area (adjoining the implant) was measured.

Micro-computed tomography (Micro CT): MicroCT scans of each sample of both types of implants were obtained with a Skyscan 1172 equipment (Kontich, Belgium) at 6 µm resolution with 800 ms exposure time, 70 kV electric voltage, 167 µA current, and a 0.5 mm thickness aluminium filter. The equipment was fitted with a 1.3 MP camera to capture high resolution 2D images that were assembled into 3D reconstructions using NRecon software supplied with the instrument.

Statistical methods: Mean values and standard deviations were calculated for bone implant contact (BIC). The mean differences of % BIC between the groups were verified through a Mann–Whitney nonparametric test, *P* value <0.05 was considered significant. Statistical analyses were carried out with the help of SPSS statistical software version 18.

Results

Clinical findings: On the whole, postoperative wound healing in all the rabbits was good. None of them exhibited any signs of wound infection or exposure. A total of 36 specimens were retrieved for histological examination.

Histological observations: All of the implants in both groups showed osseointegration and displayed a good amount of bone contact length (Figures 4 and 5). No discernible differences were noticed between both the groups. The zone of interest was 200 µm in the peri-implant area of the implants on both sides. Due to large marrow spaces in the rabbit bone, larger volume of bone contact was mostly observed in the coronal and apical portions of the implants. The MicroCT

pictures showed a three-dimensional deposition of bone in both samples (Figure 6). It was noted that possibility of new bone formation was higher in areas adjacent to old bone. The sections of implant, which were exposed to the marrow spaces, displayed either no bone deposition or very thin bone tissue. Newly formed bone was seen with lighter staining. In the surrounding areas of both types of implants, bone fragments were noticed around the implant. These could correspond to bone fragments during the osteotomy procedure. Percentage of BIC ranged from 45 to 67% in both the groups. The median value of % BIC was 58.5, MDI group (IQR 8), and control group was 57.0 (IQR 5.5) (Tables 1 and 2). The mean differences of % BIC between the groups were verified through a Mann–Whitney nonparametric test. There was no significant difference between the % bone implant contact (BIC) length of both the implants (*P* value >0 .05).

Discussion

The osseointegration potential of 3M™ESPE™ MDIs has not been studied. The MDI is a one-piece implant that simplifies the restorative phase, resulting in a reduced cost for the patient. Titanium-aluminium-vanadium alloy (Ti 6Al-4V ELI) is used for increased strength. The success of these implants led to its use in long-term fixed and removable dental prostheses (51). Conventional implant treatment requires adequate bone width and interdental space. Augmentation procedures are complex and can cause post-operative pain and discomfort for the patient and additional costs.

In human models, a 3-6-month period is needed to obtain osseointegration and animal models would need a shorter time (4-6 weeks) (30, 33). Rabbit has been used extensively to examine osseointegration and appears to be an appropriate model for studying the bone healing systems (52). The healing periods used by various authors for assessing the bone implant contact in rabbits are 2, 3, 4, 6, 8 and 12 weeks (53-57). However, the best results have been between 6 and 12 weeks

of insertion period (51, 53-55). The 6-week healing period was carefully chosen after literature search. This was in agreement with others who have reported that a 6-week period is adequate in rabbits to develop a “rigid osseous interface” (51-60).

At the bone implant interface, woven bone starts forming after the placement of implant. Lamellar bone slowly replaces this scantily organized bone. The fully developed lamellar bone which replaces the woven bone typifies a stable and lasting osseointegration (61).

Our results are in concurrence with Balkin et al. (62); they have also shown in their histology study in humans that the MDI undergoes osseointegration. They inserted one 3M™ESPE™ MDI of 1.8-mm diameter in each of two patients as a transitional implant for mandibular dentures. After a period of 4 and 5 months, the implants were trephined out for histological evaluation. The results showed that there was a close apposition of bone on the implant surfaces. The bone surrounding the implant demonstrated signs of matured healing and integrated for immediate function after 4 to 5 months of healing period.

Our study is also in concordance with the results of a removal torque study by Simon et al. (63) in immediately loaded “transitional endosseous implants” in humans. The percentage BIC for MDIs was similar to standard implants.

The surface topography also affects the BIC, Wennerberg et al. (32) measured and compared removal torque values on screw-shaped titanium implants with three surface types. The results showed that screws sandblasted with 25-µm particles of titanium and 75-µm particles of aluminium oxide exhibited a higher removal torque and interfacial bone contact than the machined titanium implants with smoother surface texture.

The surface of 3M™ESPE™ MDI is sandblasted with aluminium oxide as well as cleaned and passivized with an oxidizing acid (Technical Data Sheet, 3M ESPE) (35). The surface of Ankylos® is sandblasted and acid etched (64). Various authors have reported that surface roughness induces a variety of events in the course of osteoblast differentiation, spreading and proliferation, production of alkaline phosphatase, collagen, proteoglycans and osteocalcin, and synthesis of cytokines and growth factors (65-67). Therefore, leading to bone deposition on the surface of these implants. Yan et al. (68) demonstrated that simple surface treatments can turn the titanium surface into a bone-bonding one. With the results of our *in vitro* study by Marulanda et al. (69) on discs of both types of implants demonstrated that surface chemistry of 3M™ESPE™ MDI is conducive to growth of osteoblasts leading to bone apposition.

One of the shortcomings of our study may be the use of rabbit tibia as a model. The tibia of the rabbit is essentially hollow except the upper and lower cortical plates. This may justify lack of bone apposition on the whole implant in both experimental as well as comparator implants. However, it provides reliable information for human application as the human maxillary bone is also of a softer bone quality (36, 51).

Conclusion: The results of this study show that MDIs as well as regular implants osseointegrate in rabbits.

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Authors' contributions : JSD carried out the experiments and drafted the manuscript, RA conceived the study and helped in revising the manuscript, MM contributed to the histological preparation and data analysis, JSF participated in this study's design and overall coordination. All authors read and approved the final manuscript.

Competing interests: Jagjit Singh Dhaliwal, Rubens F. Albuquerque Jr, Monzur Murshed and Jocelyne S. Feine declare that they have no competing interests.

Ethical Approval: McGill University Research Ethics Board, Animal Use Protocol # 2012-7221. All study procedures were conducted as per McGill SOPs. All efforts were made to minimize distress in animals throughout the experiments, as well as to use only the number of animals that was essential to produce reliable scientific data.

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Table 1. Comparison of percentage BIC in both groups

Sample	3M™ESPE™ MDIs	Ankylos®Friadent (Dentsply)
1.	67	54
2.	59	67
3.	54	45
4.	51	58
5.	47	57
6.	64	49
7.	50	54
8.	60	56
9.	56	60
10.	61	53
11.	62	59
12.	61	55
13.	59	59
14.	45	51
15.	58	59
16.	54	62
17.	66	62
18.	56	57

Table 2. Descriptive statistics of the experimental and control group

BIC	3M™ ESPE™ MDIs	Ankylos® Friadent (Dentsply)
Median	**58.5**	**57**
Mean	**57**	**56.5**
Interquartile range	**8**	**5.5**
First Quartile	**53.25**	**53.75**
Third Quartile	**61.25**	**59.25**

Figure 1. Radiograph showing implants in the rabbit tibia

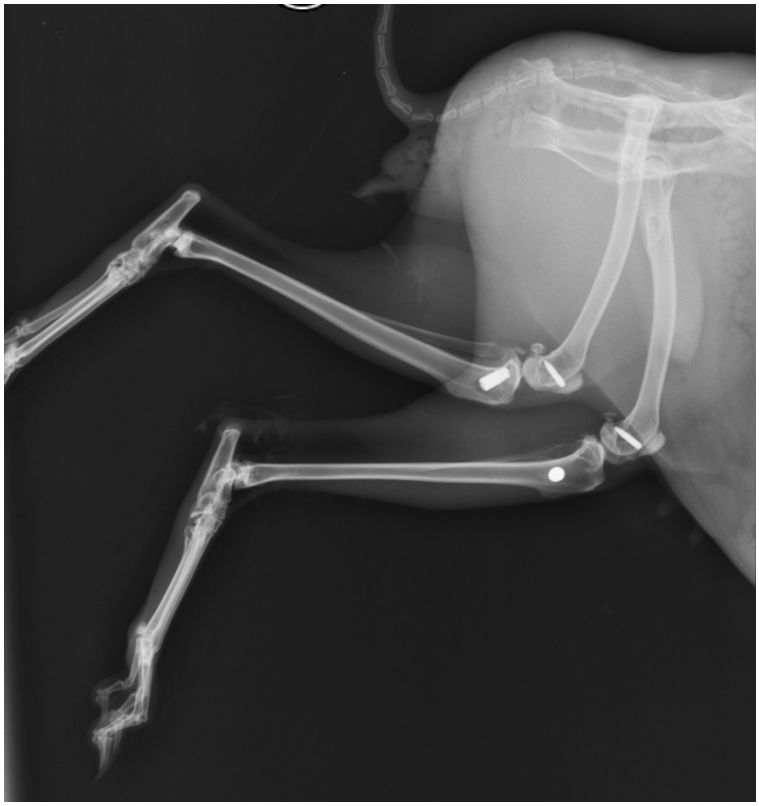


Figure 2: Leica SP 1600 saw microtome

Figure 3: Histological sections being obtained with Leica SP 1600 saw microtome

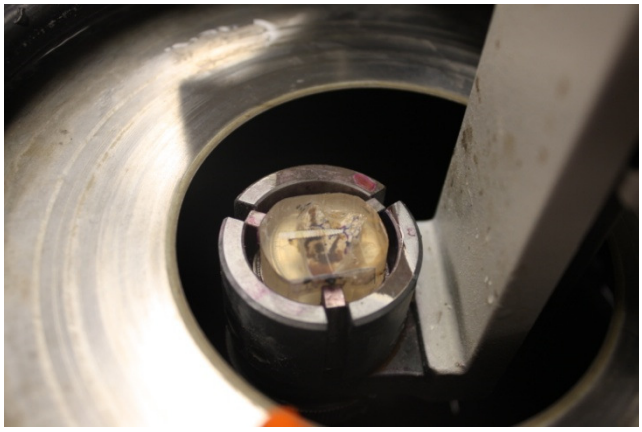


Figure 4: Histological section of Mini Dental Implant in rabbit tibia stained with Methylene blue and Basic Fuchsin

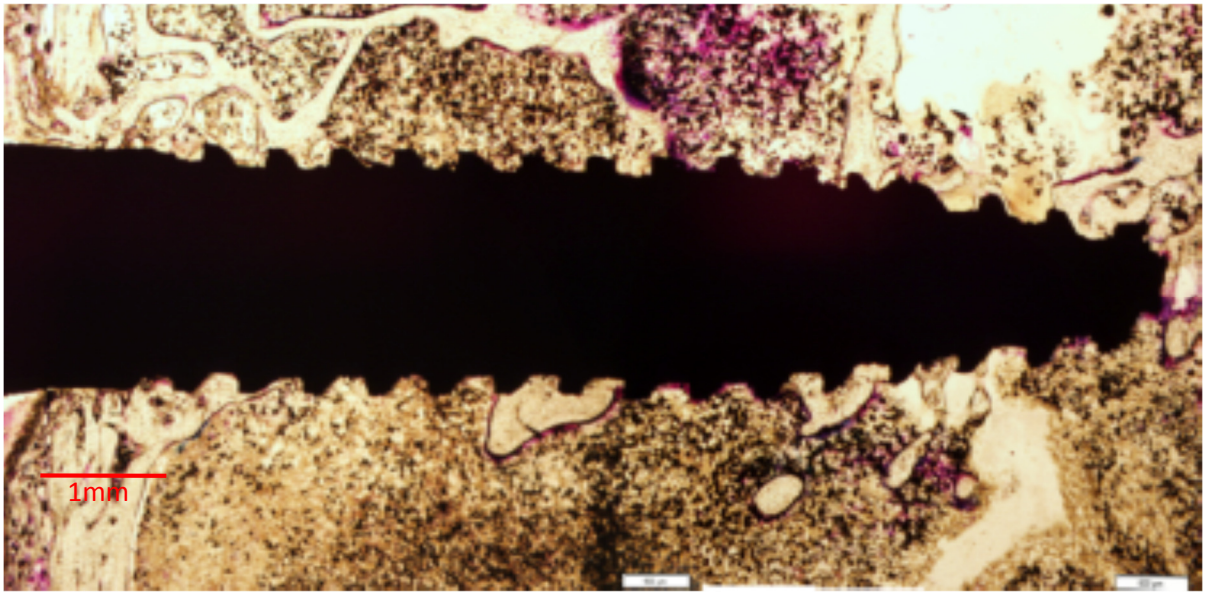


Figure 5: Histological section of standard implant in rabbit tibia stained with Methylene blue and Basic Fuchsin

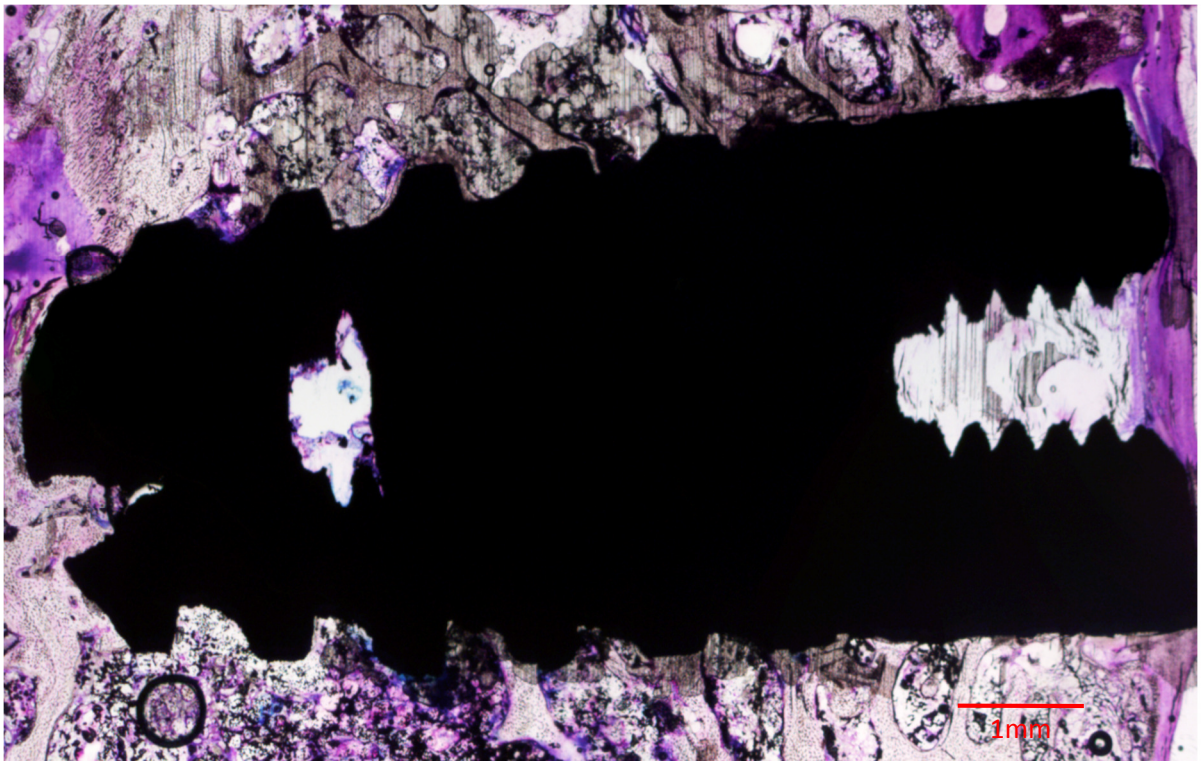
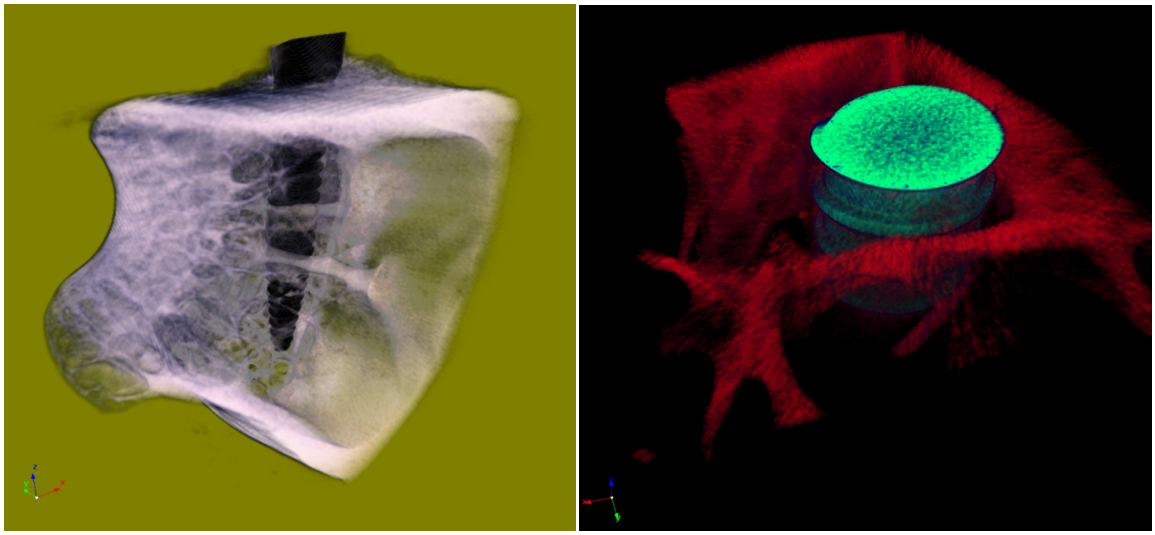


Figure 6 : Micro CT scan images of the MDIs and Ankylos® embedded in rabbit bone 6 weeks post implantation



4.5 Part III

Measuring the Elastic Modulus and Hardness of the Bone-Implant Interface in Mini Dental Implants and Standard Implants with Nanoindentation Method

Previous experiments including cell culture study, resonance frequency analysis and Bone Implant Contact (BIC) measurement revealed no significant differences between the MDIs and a standard implant.

Literature shows that a number of reasons may lead to untoward clinical outcomes e.g. ill-fitting prostheses, parafunctional habits, material fatigue, and implant design (143, 144). The mechanical properties of implant material, the quality of the newly formed interface and surrounding bone may play an important role in the development of a solid union of bone with the implant.

It has been shown that increased Bone Implant Contact, obtained by augmenting surface roughness, may not always improve biomechanical interactions with bone (28, 145). Histomorphometry may not be able to show different degrees of mineralization. The stability of the implant could also be elicited by greater bone area instead of rapid mineralization of bone.

Implants require sufficient mechanical features to withstand functional occlusal loads (146). Qualitative characteristics of osseointegration may be explained by nanoindentation of bone around the implants (125), as nanoindentation is an appropriate method to look into the anisotropic configuration of bone (147) and may be used to measure different mechanical properties of the implant materials, as well as the surrounding bone. We sectioned each implant embedded in the resin block into two parts, one side for histomorphometry and the other for depth sensing nanoindentation tests. The following manuscript is submitted to the *International Journal of Implant Dentistry.*

4.6 Manuscript IV

Exploring the Properties of Bone Surrounding Osseointegrated Mini Dental Implants and Ankylos® Implants using Nanoindentation

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Abstract

Background: Single-piece 3M™ESPE™ Mini Dental Implants (MDIs) are manufactured from Ti6Al-4V ELI. These mini dental implants are used for overdentures and have many advantages over regular and wider diameter implants. It is vital for these implants to be made of a biomaterial that can bear high levels of stress generated by masticatory forces.

Objective: To measure the elastic modulus and hardness of the implant and bone near the bone implant interface in Mini Dental Implants and standard implants via the nanoindentation method.

Methods: Nine clinically healthy New Zealand white rabbits were used for the study using the heads of tibia/femur of the animals for the implantation of samples. Each animal received a total of 4 implants (2 experimental and 2 regular implants). Standard surgical procedures were used for osteotomy according to manufacturer protocols. The animals were euthanized after 6 weeks and specimens were removed *en bloc*. The specimens were embedded in a light curing methyl methacrylate resin and prepared by sectioning with a Leica SP 1600 saw microtome. The resulting specimen was glued to an acrylic plate, then ground with the help of CarbiMet® abrasive papers of 240 to 600 grit. The specimens were then polished with the help of SiC abrasive papers and polished using TexMet® 1500 cloth and diamond paste. The elastic modulus (E) and hardness (H) of the bone surrounding the implants were measured by depth-sensing nanoindentation (Hysitron Inc. TI950 Triboindenter) on implant surface, bone implant interface and bone beyond 200µm at three zones; coronal, middle and apical.

Results: There were no statistically significant differences in the elastic modulus (p>0.05) between the two groups in all three locations (implant surface, near bone implant interface and bone at 200µm) in all the three zones. There were statistically significant differences between the two

groups in the hardness of bone at 200µm (p<0.05). However, the differences were not significant in the implant surface and bone implant interface locations (p >0.05). The differences were not significant in the hardness and elastic modulus of bone near the interface and at 200µm in the Ankylos® and MDIs.

Conclusion: The mechanical properties of 3M™ESPE™ MDIs and a standard dental implant have been found to be similar.

Keywords: Mini Dental Implants, Mechanical Properties, Nanoindentation

Background

Titanium is considered to be one of the best metals used for dental implants due to its biocompatible nature. This property helps in the osseointegration (1) and load bearing capability of the dental implants needed for prosthetic rehabilitation (2). Adequate mechanical properties are required for an implant to withstand occlusal loads (3). However, the implants may not function as expected and may have complications such as fixture fracture and bone loss. Various factors may be responsible for these unwanted outcomes; for example, parafunctional habits, ill-fitting prosthesis, material fatigue as well as size and design of the implant (4-6). In such circumstances, it may be necessary to use a biomaterial, which can tolerate high levels of forces generated by the factors listed above. Higher elastic modulus means increased stiffness and distributed force transfer (7). Biphasic (α+β) titanium alloys such as Ti6Al4V have shown higher mechanical properties than cpTi alloys (8). Therefore, currently, implant manufacturers have resorted to using titanium alloys as implant biomaterials (9).

Single-piece 3M™ESPE™ MDIs used for implant overdentures are manufactured from Ti6Al-4V ELI, a titanium alloy which is specified by the American Society for Testing and Materials (ASTM) F 136: Standard Specification for Wrought Titanium-6 Aluminium-4 Vanadium ELI (Extra Low Interstitial) alloy for Surgical Implant Applications (UNS R56401). (10).

This is a material of choice for many medical and dental applications owing to its excellent biocompatibility, good fatigue strength and a low elastic modulus, particularly for implantable components. The ELI grade has superior damage tolerance, including fracture toughness and fatigue crack growth rate (11).

The nanoindentation method has been used for measuring the mechanical properties (elastic modulus and hardness) of bone surrounding the implants at the micro structural level and has been published by many authors. This is a characterization technique with a spatial resolution of more than 1µm (12). A number of researchers have studied these mechanical properties of bone around dental implants. Zysset et al. (13) reported that the values of elastic modulus were higher in the cortical than in the trabecular bone. Rho et al. (12) reported that the values of elastic modulus were higher in the interstitial lamellae than in the osteonal bone in humans. Huja et al. (14) studied endosseous dental implants (HA-coated and uncoated) implanted in the mid-femoral diaphyses of male hounds. After 12 weeks *in vivo*, the micro-hardness of bone increased phenomenally at a distance of 200-600µm from the implant bone interface. Chang et al. (15) studied titanium dental implants in swine alveolar bone and one month later noticed a gradient in bone modulus within 200µm of the implant followed by a plateau and increase in modulus at a distance of 1000µm and greater. The indentations can also be performed at the bone implant interface for studying bone quality. (7, 13, 14, 16, 17). There is very limited literature studying the biomechanical properties for example, hardness/elastic modulus of bone integrated to 3M™ESPE™ MDIs.

Hypothesis: It was hypothesized that the mechanical properties of implant and bone surrounding 3M™ESPE™ MDIs would be similar to a standard implant.

Objective: The aim of this study was to measure the elastic modulus and hardness of the implant and bone near the bone implant interface in Mini Dental Implants and standard implants via the nanoindentation method.

Materials and Methods

Animal Model: Nine clinically healthy New Zealand white rabbits were used for the study. The animals were housed in the Central Animal House facility. The heads of tibia/femur of the animals were used for the implantation of samples. Rabbits' tibia and femur have been widely used as an animal model by various other authors to study osseointegration of dental implants (18-27). Each animal received two implants on both the hind limbs (right and left tibia/femur heads) quasi-randomly (the heads of tibia and femur have been chosen to get the maximum bulk of bone. Therefore, each animal received a total of 4 (2 experimental and 2 regular) implants.

Surgical Procedure: The procedures had been approved by the institutional animals' ethics committee of McGill University, Canada. Standard surgical procedure for 3M™ESPE™ MDIs and Ankylos® implants was used for osteotomy respectively, according to manufacturer protocols. The animals were euthanized after 6 weeks of healing. It has been discovered by various authors that a 6-week healing period in rabbits is sufficient to develop a "rigid osseous interface" (28-30). The implants along with their surrounding bone were excised with a surgical saw immediately after the euthanasia. The excess tissue was dissected and the specimens were removed *en bloc* with a margin of surrounding bone of about 5-10 mm. The specimens were immediately deposited into the 10% formaldehyde solution.

Specimen Preparation: The specimens were dehydrated in the ascending graded ethanol solution, kept in a pre-filtration solution for 3 hours at room temperature and then in the filtration solution at 4^0C for 17 hours. The specimens were then embedded in a light curing resin Technovit 9100 NEW (Kulzer & Co., Wehrheim, Germany) polymerization system based on methyl methacrylate. These blocks were cut with a low speed saw under water along the lateral surface of

the implant (21, 31). The specimens were prepared and sections were obtained using the Leica SP 1600 saw microtome. The acrylic block was mounted onto the object holder of the microtome. The height of the object was adjusted until the surface of the object was slightly above the upper edge of the saw blade. The surface of the block was trimmed to obtain a plane surface. During the sawing process, the water flow was adjusted so that the water jet would land on the edge of the saw blade. This built-in water-cooling device prevents overheating of the object and removes sawdust from the cutting edge and thus prolongs the life of the saw blade. The most favorable feed rate was determined and, after trimming, the first undefined slice was removed from the saw blade. Next, the desired section thickness was selected after considering the thickness of the saw blade and added to the desired thickness of the final section. The saw blade was adjusted to obtain a sample in such a way that the implant would be sectioned into two halves. One side of the block was used for making a histology slide and the other side was glued to an acrylic plate for polishing followed by nanoindentation testing.

Specimen polishing procedure: The specimens thus obtained were ground with the help of CarbiMet® abrasive papers (Buehler Canada) starting with 240-320 grit and with finer abrasives of 400 to 600 grit papers. The specimens were then polished with the help of SiC abrasive papers starting with 1500 grit up to 12000 grit. Further polishing was performed using TexMet® 1500 cloth, (Buehler Canada) using MetaDi® II 3µm diamond paste and MetaDi® fluid as lubricant. Finishing was completed using colloidal silica on a MasterTex® polishing cloth to remove scratches.

Depth-sensing nanoindentation testing: This technique was used to measure the mechanical properties of bone. A Hysitron Inc. Triboindenter (TI950) was used to test the samples (Figure 1).

The elastic modulus (E) and hardness (H) of bone surrounding the implants was measured at three zones: coronal, middle and apical. At each zone, three locations, the implant surface, near the bone implant interface and bone beyond 200µm were tested (Figure 2). The light microscope of the nanoindentation system was used to distinguish and spot the bony tissue so that the values were taken from bony tissue and not from other tissue or resin.

A low load of 2000µN for all indentations was used and the indentations were positioned (precise to ±1µm) using the system's optical microscope. These indentations were depth resolved which means that the depth and load were measured throughout the indentation process. We used a trapezoidal loading function (with 5 seconds loading, 5 seconds unloading and a hold segment of 2 seconds) for the measurement. The un-loading force–displacement curve was used to calculate the reduced modulus using the Oliver and Pharr method (32) as this is the most widely used method to extract the Elastic Modulus (E) and Hardness (H) from the indentation curves. In order to accurately evaluate the area of contact between the tip and the sample as a function of depth, the tip area function was calibrated over the whole measurement range using a fused quartz reference sample of known modulus (72 GPa). From each curve, the reduced modulus (GPa) and hardness (GPa) of bone tissue were computed using Hysitron TriboScan software and the elastic modulus E (GPa) was then calculated according to the following formula: $\frac{1}{E_r} = \frac{1-V^2}{E} + \frac{1-V_i^2}{E_i}$.

E_r is the reduced modulus (GPa), V is the Poisson's ratio for cortical bone (0.2), E_i and V_i are the elastic modulus and Poisson's ratio for the diamond indenter (1140 GPa and 0.07 respectively).

Scanning Electron Microscopy: Scanning electron microscopy (SEM) was performed on the 3M™ESPE™ MDI and Ankylos® disk surfaces. Samples were sputter coated and viewed with a Carl Zeiss AG-EVO® 40 series scanning electron microscope.

Optical Microscopy: A Carl Zeiss Axio Scope A1 Light Microscope was used. 18 polarized light images were collected at 10x magnification and stitched together using an image editor software (Adobe Photoshop).

Statistical methods: Mean values and standard deviations were calculated for hardness (H) and indentation elastic modulus (IM). Univariate analysis was done for all the evaluations. ANOVA was used to analyze the differences between the two implants. For all statistical tests $P<0.05$ was considered significant. Statistical analysis was performed with the help of SPSS statistical software version 17.

Results

The mean hardness in the three implant surface zones was 3.43 (σ 1.3) for Ankylos® and 3.4 GPa (σ 0.3) for the MDIs. The mean elasticity modulus; Ankylos® was 83.99 GPa (σ 38.07) and MDIs 104.15 GPa (σ 17.08), respectively (Table 1).

Mechanical properties near the bone implant interface in both groups: mean hardness 0.89 GPa (σ 0.8) and mean elasticity modulus 6.06 GPa (σ 5.0) in Ankylos®; mean hardness 0.88 GPa (σ 0.63) and elastic modulus 13.03 GPa (σ 9.1) was observed in the MDI group (Table 1).

Bone at 200µm distance from the implant: mean hardness for Ankylos® group was 1.25 GPa (σ 0.47), elastic modulus 21.11 GPa (σ 11.02) whereas hardness was 0.69 GPa (σ 0.4), 13.5 GPa (σ 7.4) elastic modulus for the MDIs.

Statistical differences between the two implant groups: Implant surface hardness $p=0.9475$, elastic modulus implant $p=0.1665$. Near the bone implant interface; hardness $p=0.9760$, elastic modulus $p=0.0616$.

Bone at 200um; hardness p=0.0119, elastic modulus p=0.1042 in different zones. Differences in hardness between the bone in two locations: Hardness of bone near interface and at 200µm Ankylos® p=0.2444, hardness of bone near interface and at 200µm MDI, p=0.4436.

Differences in Elastic Modulus in the Ankylos® group in three zones between the bone near the interface and bone at 200µm p=0.0018, the MDI group Elastic Modulus near the interface and bone at 200µm p=0.9057.

There were no significant statistical differences in the elastic modulus ($p > 0.05$) between the two groups in all the three locations implant surface, near bone implant interface and bone at 200µm. There were statistically significant differences between the two groups in the hardness of bone at 200µm ($p< 0.05$). However, the differences were not significant in the hardness and elastic modulus of bone near the interface and at 200µm in the Ankylos and MDIs.

Scanning electron microscopy was used in SE mode under 10 kV acceleration voltage for producing the images to observe the surface topography and it showed increased surface roughness in the 3M™ESPE™ MDIs compared with Ankylos® (Figure 3).

Discussion

Currently, focus is increasingly on the stability of the implant, which is able to withstand strong loading forces. The mechanical properties and surface characteristics of the implant may influence the bone deposition around it. We focused on the comparison of mechanical properties of bone surrounding 3M™ESPE™ MDIs and a standard implant (Ankylos®) with the help of a nanoindenter. The tests were conducted on one half of the embedded samples. The biomechanical properties of

bone tissue in both the implant groups were similar in all the three regions assessed. Ti6Al-4V ELI is the alloy used for manufacturing of 3M™ESPE™ MDIs.

The results of this study suggest that the biomechanical properties of this Mini Dental Implant and the bone around it are similar to a standard implant. Our findings are similar to Oyen et al. (33) who studied the specimens using a viscous-elastic-plastic indentation model and reported that there was an increase in the modulus with increased distance from the bone-implant interface. Our results have shown lower values than the study of Kanie et al. (34), who investigated the physical properties of two mini implants, wherein they compared MDIs with a an MTI (Mini Transitional Implant). This can be due to differences in tip area function calibration (15) or bone debris trapped between the tip and the surface prior to indentation testing. The presence of a mechanically damaged layer formed during the cutting, mounting, grinding and polishing steps can significantly affect the measured mechanical properties. The polishing of these samples is difficult due to different physical properties of the sample materials, for example resin, bone and implant.

The final colloidal silica polishing step is critical to removing this damaged layer. The hardness of bone was similar near the bone implant interface in both the groups, whereas elastic modulus was significantly higher at the bone implant interface in the MDI group. According to a systematic review by Wennerberg et al. (35), the surface characteristics of the implant may influence enhanced outcomes at the bone implant interface. It may be due to the increased surface area of MDIs and the rough surface topography, which was confirmed on scanning electron microscope pictures of the MDIs compared with Ankylos®. The surface treatment of 3M™ESPE™ MDIs involves sandblasting with aluminium oxide and passivation with an oxidizing acid; Ankylos® surface is sandblasted and acid etched.

Conclusion

Depth-sensing nanoindentation seems to be an appropriate method for recording the mechanical properties of bone around the dental implants *in vitro*. The mechanical properties of 3M™ESPE™MDIs and standard dental implants have been found to be similar.

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Competing interests: Jagjit Singh Dhaliwal, Rubens F. Albuquerque, Thomas Schmitt, Etienne Bousser and Jocelyne S. Feine declare that they have no competing interests.

Ethical Approval: McGill University Research Ethics Board, Animal Use Protocol # 2012-7221. All study procedures were conducted as per McGill's SOPs. All efforts were made to minimize distress in animals throughout the experiments, as well as to use only the number of animals that was essential to produce reliable scientific data.

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Table 1: Mean values and standard deviation of Hardness and Elastic Modulus at different locations of MDIs and Ankylos®

Locations	Hardness (Mean and SD)				Elastic Modulus (Mean and SD)			
	Ankylos®	σ	MDI	σ	Ankylos®	σ	MDI	σ
Implant Surface	3.43	1.31	3.40	0.31	83.99	38.07	104.15	17.08
Bone implant interface	0.89	0.76	0.88	0.63	6.06	5.0	13.03	9.12
Bone @200µm	1.25	0.47	0.69	0.36	21.11	11.02	13.5	7.36

Figure 1. Hysitron Inc. Triboindenter (TI950)

Figure 2. Photograph of Sectioned and polished sample picture of MDI with areas marked for Nanoindentation testing

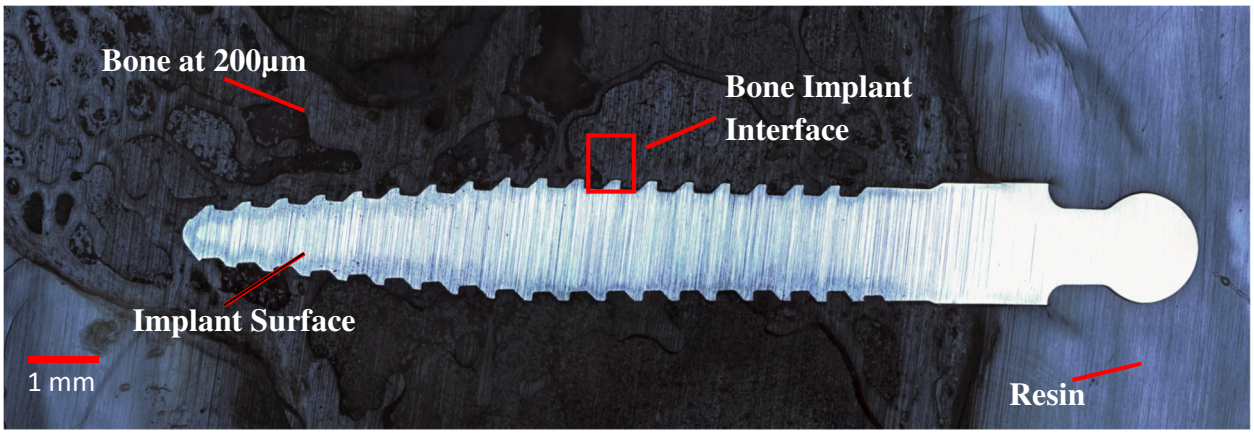
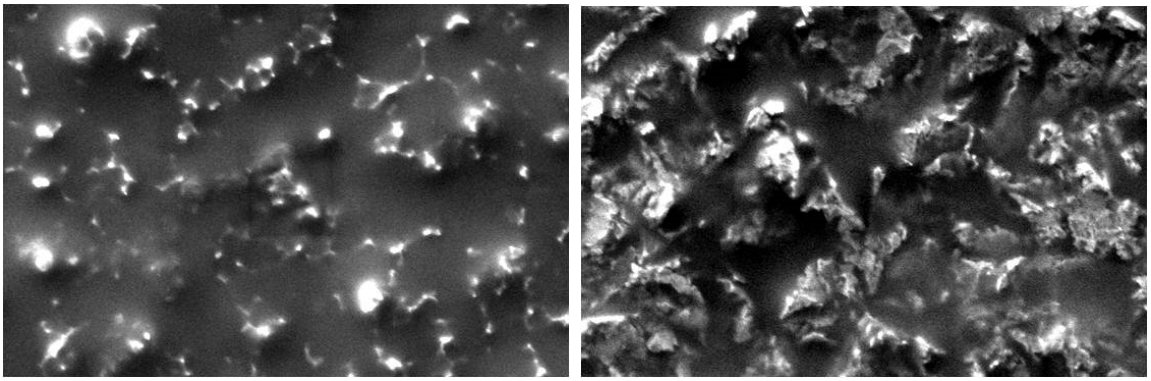


Figure 3. Implant surfaces topography under Scanning Electron Microscope



Ankylos® **3M™ESPE™MDIs**

Chapter five: General Discussion

5.0 Discussion: Increasing trends suggest that the use of minimally invasive dentistry is increasing and should be relevant to implantology, as well. Thus, MDI use is in concordance with minimally invasive dentistry. The single piece Mini Dental Implants (MDIs) do not require a separate abutment and have been interchangeably referred to as Narrow Diameter Implants (NDIs) or Small Diameter Implants (SDIs). They make the restorative phase simpler and lower in cost for patients.

According to the 2013 ITI consensus statement, single piece MDIs with a diameter of 1.8-2.9 mm have shown a mean survival rate of 94.3% for up to 6 years of follow-up (35, 148-150). These have been used as overdenture treatment for edentate populations and as replacements of single anterior teeth. The MDI implant was originally planned and designed for stabilizing interim dentures during the healing phase of standard sized implants. However, there is inadequate evidence on the success rates of all Narrow Diameter Implants (NDIs). While narrow diameter implants have many indications, information on their clinical performance is scarce.

MDIs are a good option for patients with atrophic edentulism. Selected patients suffering from systemic conditions can also benefit, as they can receive an immediate overdenture with MDI support/retention. The 3M™ESPE™ MDI was originally fabricated for denture stabilization during the healing period of regular implants, orthodontic anchorage and interim fixation for transplanted teeth (11).

Traditional implant treatment necessitates adequate amounts of bone width and interdental space. Even though techniques are available to augment insufficient bone, they are expensive and painful for the patient.

In this series of studies (*in vitro* and *in vivo*) we compared 3M™ESPE™ MDI with a standard implant (Ankylos®).

5.1 *In vitro* Study:

The 3M™ESPE™ MDI implant surfaces are treated to enhance surface area for promoting a sturdy osseointegration. The surface treatment involves sandblasting with aluminium oxide and subsequent cleaning and passivation with an oxidizing acid (135). The scanning electron microscopic observations confirm that the surface area of the implants are sufficiently roughened to enhance bone-to-implant contact (135). Sandblasted surfaces have been shown to promote peri implant osteogenesis by augmenting the metabolic activity of osteoblasts (151). This property has been shown to help in orientation and locomotor activity of some cell types and influence cell shape and function directly (152, 153).

Several other authors have reported that surface characteristics and degree of roughness influence the healing process positively by encouraging cellular responses and cell surface interactions (154-156).

Our *in vitro* study comparing the MDI surface with an established standard implant surface has also shown that the surface texture of MDIs is more conducive to osteoblastic cell adherence and proliferation compared with a standard implant. There was an obvious difference in the level of biocompatibility between the two types of implant surfaces; the 3M™ESPE™ MDI showed higher cell numbers and increased deposition of calcium phosphate minerals in comparison to Ankylos®. The increased cell number may be the primary reason why cultures grown on 3M™ESPE™ MDI deposited more minerals in comparison to that grown on Ankylos®. Based on this theory, it is

likely that MDI has a superior surface quality to promote osseointegration. However, this needed to be tested *in vivo*.

5.2 *In vivo* study:

The primary stability is important for achieving osseointegration. Excessive micro motion is thought to cause development of a fibrous encapsulation, instead of osseointegration (157).

Since the MDIs are mostly immediately loaded, it is important to measure the initial stability. It is possible to measure stability of standard implants by using Osstell ISQ device with SmartPegs supplied by the company. However, the SmartPeg attachment for one piece implants is not manufactured by the company. Therefore, we developed a customized SmartPeg for the purpose of measuring MDI stability and compared it with a standard implant. The MDI has shown good implant stability equal to the comparator implant. The resonance frequency assessment with a customized SmartPeg would be a useful tool to provide clinically useful information about the condition of the bone-implant interface of 3M™ESPE™ MDIs. Frequently, implant failures are associated with biomechanical reasons and, to a great extent, timely implant stability assessment can reduce these failures. Studies have indicated that a higher resonance frequency assessment value results in greater success in implant treatment and lower risk of failure in the future. On the other hand, lower values may indicate greater risk for implant complications. We found no studies in the literature that provide data based on resonance frequency measurements for MDIs. The exact RFA threshold values for MDIs may have to be identified with more studies conducted *in vivo*.

Even though the MDIs have a smaller surface area than standard implants, our histological study has shown that the BIC of MDIs is as good as standard implants. According to English et al (158), the surface area of five MDIs is equivalent to two regular implants of 3.75mm diameter with equal length. The bone to implant contact percentage was as good as that of the Ankylos® implant. Our

findings are similar to a histological study by Balkin et al. (159), in which they used trephines to take out the MDI along with surrounding bone in humans; however, the sample size was too small to draw any conclusions. In another small histological study conducted at Loma Linda University, osseointegration of MDIs was assessed in miniature swine at 3 and 6 months for histomorphometric evaluation of BIC. It was concluded that MDIs are able to achieve osseointegration at 3 months post insertion (160).

When comparing the dimensions of MDIs and regular implants, the volume of standard implants is roughly 4 times that of an MDI. It has been shown that the larger volume may actually be a deterrent in the angiogenesis and osteogenesis, as it may become a physical barrier for cellular growth and cytokine activity (161). It has been reported that the shorter length MDIs /SDIs have a higher failure rate than do longer implants (162).

Mini Dental Implants are smaller than traditional dental implants and bear high occlusal loads. Therefore, these implants are manufactured with Aluminum (Al) and Vanadium (V) (Ti-6Al-4V) for greater strength and fatigue resistance. The 3M™ESPE™ MDIs are fabricated from Ti 6Al-4V ELI (Grade 23) titanium alloy, which is a standard alloy for surgical implant applications. The increased strength and other properties make this alloy tougher than pure Titanium (135). The mechanical properties and surface characteristics of the implant may influence the deposition of bone. We compared elastic modulus and hardness of implant material of 3M™ESPE™ MDIs and a standard implant (Ankylos®) with the help of a depth-sensing nanoindentation instrument. It is important to investigate properties of the bone at the interface and surrounding the implant, which needs to be strong enough to be able to withstand strong loading forces.

The biomechanical properties of the Mini Dental Implant and the bone around it were found to be similar to the standard implant. Our findings are similar to Oyen et al. (163), who studied the specimens using a viscous-elastic-plastic indentation model and reported that there was an increase in the modulus with increased distance from the bone-implant interface. Our results have shown lower values than the study of Kanie et al. (164), who investigated the physical properties of two mini implants, wherein they compared MDIs with an MTI (Mini Transitional Implant). This can be due to differences in tip area function calibration (129) or in differences in the surface preparation methods of the cross-sections prior to indentation testing. Indeed, the presence of a mechanically damaged layer formed during grinding and polishing steps can significantly affect the measured surface mechanical properties. The final colloidal silica polishing step is critical to removing this damaged layer. The hardness of bone was similar near the bone implant interface in both groups, whereas elastic the modulus was significantly higher at the bone implant interface in the MDI group.

5.3 Strength of this Study: A major strength and uniqueness of this research is that osseointegration of 3M™ESPE™ MDIs was measured with a variety of methods on the same implant samples. This included an *in vitro* cell culture experiment and *in vivo* animal study. The same comparator implant surface was used *in vitro* and *in vivo* to corroborate the results. This was important because many factors play a role and may influence the osseointegration process. Therefore, it was essential to test the same samples in a variety of ways in order to understand bone healing around implants.

5.4 Limitations of the studies and future research

The rabbit is a good animal model for dental implant research. However, it has its limitations, as rabbit bones are small, limiting the size and number of implants that can be placed. Rabbit bones also have a large bone marrow space that does not mimic human bone structure. The international standard for the biological evaluation of medical devices has recommended a total of six implants (3 test and 3 control) per rabbit (International Standard ISO 10993-6, 1994). However, we can use double the number of implants for sheep, dogs, goats and pigs. We used four implants per rabbit, as it was not possible to find more available cortical bone other than near the joints. Additionally, we were not able to test these implants under functional loading in rabbits as the force distribution may have influence on osseointegration in the narrow diameter implants.

Future research may include randomized clinical trials with long-term follow-ups to determine whether MDIs and standard sized implants will demonstrate similar Osseointegration characteristics under function and in patient populations.

Chapter six: Conclusions

6.0 Conclusions: This body of *in vitro* and *in vivo* evidence suggests that 3M™ESPE™ MDI implants have the potential to undergo osseointegration, and are comparable to a standard-sized well established implant. Therefore, it appears that these may be used successfully in humans for denture stabilization.

The following specific conclusions can be drawn from this research:

- A good response of osteoblastic cell attachment on the MDI surface.
- Surface property may play a significant role in initial attachment of cells and their proliferation ability, which may help in providing improved osseointegration of 3M™ESPE™ MDIs.
- Resonance frequency assessment (ISQ measurement) of these single-piece 3M™ESPE™ MDIs is possible with the help of a custom made SmartPeg device.
- 3M™ESPE™ MDIs attain primary and secondary stability at the same levels as standard implants in the rabbit tibia.
- MDIs osseointegrate as well as regular implants in rabbits.
- The mechanical properties of 3M™ESPE™ MDIs and standard dental implants are similar.
- The MDI system seems to have acceptable results *in vitro* and *in vivo*.

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