The Effects of Osteoporosis on Osseointegration in the Rat Maxilla

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Keywords

Osteoporosis, Rat, Maxilla, Dental implant, Osseointegration, Rough surface implant, Smooth surface implant, Osteocytes, Titanium, SEM, Acid etching, Micro CT, Tibia, Ovariectomy.
Abstract

Osseointegration has been defined as a direct bone-to-implant contact without interposed soft tissue. A rigid, functional fixation of dental implants within the host bone site achieved by osseointegration is necessary to provide long-term anchorage with the ability to support masticatory function. The osseointegration process and consequent clinical success of dental implants becomes more challenging in compromised clinical situations like osteoporosis. However, researchers still do not fully understand the biological mechanisms involved in osseointegration, and the consequential effects of conditions like osteoporosis on this process remains obscure. Osteoporosis is a condition characterised by decreased bone mineral density and a deterioration of bone microarchitecture, resulting in compromised bone quality. Therefore, osteoporosis is an interesting model to study the effect of compromised bone quality on osseointegration. Additionally, the design, chemical characteristics and topography of the implant surface is known to influence peri-implant tissue healing and subsequent osseointegration, but whether such changes to the implant surface can overcome the disadvantages resulting from the osteoporotic conditions warrants further study. The aim of this project therefore was to observe the effects of two factors: osteoporosis and implant surface topography, on osseointegration.

The ovariectomised rat is the most commonly used model to simulate human postmenopausal osteoporosis and has been selected for use in this study. The existing literature however remains inconclusive as to whether the jaw bone is affected by osteoporosis. The first part of this project aimed to determine whether the rat maxilla could be an appropriate model for osteoporosis. In order to obtain a comprehensive understanding of changes in the maxilla after ovariectomy, three dimensional Micro CT together with histological analysis was used to analyse changes in the maxilla 8, 12, 16 and 20 weeks post surgery. Significant changes were observed in the rat maxillae as early as 12 weeks following surgery. The results also confirmed that estrogen deficiency affected the rat jawbone resulting...
in compromised bone quality, thus establishing the rat maxilla as an appropriate model to explore the bone–implant healing process in osteoporosis.

The second part of the project examined the effect of osteoporosis on dental implant healing. Customised titanium implants (2×3mm) were placed bilaterally in the maxillary first molar area of Sprague-Dawley rats. Every animal received 2 implants. The animals were subsequently sacrificed up to 56 days post-surgery and resin-embedded sections of the implant and surrounding maxilla were prepared for histological and histomorphometric analyses. The percentage of bone-implant contact (%BIC) and bone area in the implant thread (%BA) were used to determine the effect of osteoporosis on osseointegration. The results showed that new bone formation around the implant increased significantly by day 14, as measured by the percentage of bone-to-implant contact (%BIC) and new bone area (%BA) in the implant thread chambers (55.1 ± 8.9% and 63.7 ± 7.7% respectively). There was a further significant increase between days 14 and 28 (p<0.05), however no significant differences were found between days 28 and 56 in either the %BIC or %BA. These results confirmed that the mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research and osseointegration following implant placement.

Successful dental implant treatment relies on the process of osseointegration whereby the titanium implant is bound to bone tissue without any intervening soft tissue. However the biological mechanisms responsible for the osseointegration process have not been fully elucidated, especially with regard to the possible role of osteocytes. Moreover, the relationship of osteoblasts and osteocytes with the implant surface and the ultrastructural details of the bone-implant interface are not fully understood. A method which can visualise the ultrastructure of the implant-bone interface in three-dimensions (3D) would therefore be beneficial in facilitating the study of the relationship between bone cells and the implant surface. The third part of this project has been designed to visualize the ultrastructural relationship between osteocytes and the titanium implant surface following osseointegration in vivo. Healthy female Sprague Dawley rats were used for this research. The animals were sacrificed 8 weeks after implantation and undecalcified tissue sections were prepared. Resin-
cast samples were subsequently acid-etched with 37% phosphoric acid prior to examination using scanning electron microscopy (SEM). The results showed for the first time that osteocytes and their dendrites were directly connected with the implant surface. This suggests that osteocytes and their lacunar–canalicular network may have an important regulatory role in establishing and maintaining long-term osseointegration.

Based on the above research, the final part of this project was designed to examine the effects of osteoporosis on osseointegration in the rat’s maxilla with different surfaced implants. Moderately rough surfaced implants and smoother (machined) implants were immediately implanted into maxillary first molar mesial sockets after tooth extraction in both ovariectomised (OVX) and sham-operated (SHAM) rats. The rats were sacrificed after 3, 7, 14, 28 days healing and the bone healing process and osseointegration were evaluated by histological observation, scanning electron microscopy, and qPCR analysis. Osseointegration as measured by the percentage of bone to implant contact (%BIC), was significantly higher around rough surfaced implants in the OVX animals when compared to machined implants in OVX animals at Day 14. The %BIC in the OVX rough implant group was similar to that seen with both rough and smooth implants in SHAM animals at this time. By day 28 however, similar levels of %BIC were measured for all test groups. This suggests that the rough surface stimulated a more rapid osseointegration process in the OVX animals.

To assess the possible molecular mechanism(s) responsible for this accelerated osseointegration on the rough surface, the level of gene expression of osteoblast, osteocyte, osteoclast and inflammatory markers at day 3 and 7 were examined. At day 3 the rough surface implant significantly increased the expression of alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen (COL1A), receptor activator of nuclear factor kappa-B ligand (RANKL), tartrate-resistant acid phosphatase (TRAP) and dentin matrix protein 1 (DMP1). By day 7, the expression of inflammatory markers reduced while the expression of the bone markers increased further overall, although there were few significant differences in the levels of expression comparing the rough and machined surfaces at this
time. These molecular results correlate with the morphological (histology and SEM) observations at these time points during the early healing events leading to osseointegration.

In conclusion, osteoporosis was found to reduce the early osseointegration (as measured by %BIC) of machined implants. However, the rough surfaced implant appeared to trigger a cell response that able to compensated for the estrogen deficient conditions, resulting in %BIC similar to that seen in SHAM animals during early osseointegration. However, by the time that the peak BIC% was achieved at 28 days post placement, this difference was not maintained as there was no significant difference in %BIC between the OVX and SHAM groups regardless of the implant surface used in the long-term (day 28).

**Conclusion**

These studies demonstrated that the rat maxilla was not only a suitable model for osteoporosis research, but also an appropriate model for dental implant research. Osteocytes and the lacunar–canalicular network were shown for the first time to be intimately involved in the osseointegration process, with the dendrites found to be in directly contact with the implant surface. Osteoporosis was found to negatively affect implant healing in the earlier stages, and in this process a micro-rough surfaced implant was able to compensate for the negative impacts of osteoporosis.
List of Publications

Published or accepted manuscripts relevant to the work performed in this PhD:


Manuscripts in preparation relevant to the work performed in this PhD:


5. Zhibin Du, Nishant Chakravorty , Saso Ivanovski, Yin Xiao “The emerging roles of osteocytes in osseointegration.”

Other publications


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>OP</td>
<td>Osteoporosis</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Trabecular bone volume fraction</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>Trabecular thickness</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>Trabecular number</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>Trabecular separation</td>
</tr>
<tr>
<td>Conn.Dens</td>
<td>Connectivity density</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of interest</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylmethacrylate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>MAR</td>
<td>Mineral apposition rate</td>
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<tr>
<td>BIC</td>
<td>Bone to Implant Contact</td>
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<td>BCR</td>
<td>Bone contact ratio</td>
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<tr>
<td>BA</td>
<td>Bone area</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>OVX</td>
<td>Ovarietomy</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>COL1A</td>
<td>Alpha-1 type I collagen</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>DMP1</td>
<td>Dentin matrix protein 1</td>
</tr>
<tr>
<td>SOST</td>
<td>Sclerostin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>BSE</td>
<td>Backscatter electron emission</td>
</tr>
<tr>
<td>LCN</td>
<td>Lacunar–canalicular network</td>
</tr>
</tbody>
</table>
Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature: __________________________

Date: September 2015
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Chapter 1 Introduction
1.1 Background

A dental implant is an artificial tooth root replacement, which is surgically placed into the jaw bone to support single or multiple tooth restorations, in order to improve the function and quality of life for the recipient. Commercially pure titanium (Ti) is widely used as a dental and orthopaedic metallic implant material due to its high biocompatibility [1, 2], good resistance to corrosion, and lack of inflammatory response in peri-implant tissues [2-4]. Other materials like tantalum [5], niobium [6], zirconium [7] and hafnium [8] have also been proposed as substitutes for titanium. In 2009, approximately 30,000 implants were placed in Australian patients ranging from 20 to 80 years of age. Implant usage has doubled in the last five years and the use of implants is increasing by approximately 15% each year. The dental implant industry has become one of the fastest growing industries in the world, and currently there are more than 1300 different types of implants available for clinical selection [9]. Thus, the replacement of missing teeth with endosseous implants for the rehabilitation of edentulous or partially edentulous patients has become the standard of care for tooth replacement [10].

What is Osseointegration?

The major breakthrough of osseointegration came from Per-Ingvar Brånemark. In 1952, the Swedish professor began his work on tissue integrated prostheses. His objective was to understand the healing and reparative capacity of hard and soft tissue in order to obtain a predictable tissue response to implant therapy. The studies in the early 1960’s were the first to describe the phenomena of osseointegration since the titanium chambers inserted into animals could not be removed from the bone. Specifically, it was noted that chambers were “inseparably incorporated” into the bone. Microscopic examination revealed bone had actually grown into the surface pores of the titanium. In separate studies on the healing and anchorage stability of titanium tooth root implants, Brånemark also found that titanium fixtures implanted into the marrow cavity would form a shell of cortical bone directly onto the implant surface [11]. This work resulted in the design of titanium dental implants and the concept of osseointegration.
The success of implants is dependent on the interface between titanium and bone. According to the concept of osseointegration first reported by Brånemark [12], osseointegration has been defined as a direct bone-to-implant contact without interposed soft tissue (Fig.1). This kind of rigid functional fixation within the host bone site assures that dental implants can provide long-term anchorage to support masticatory function. Therefore, the widespread use of dental implants in clinical practice has profoundly changed the possibilities for oral rehabilitation [13]. However, the concept of "osseointegration" of titanium implants is mainly based on clinical evaluation and light microscopic (LM) observations [14]. Morphologic observation is still the preferred way to evaluate the process of bone formation during osseointegration. Using undecalcified ground sections for measuring the bone-implant-contact (BIC) or bone contact ratio (BCR) and torque tests for measuring the biomechanical anchorage are the two most-commonly used quantifiable methods for determining the amount of osseointegration [15].

**Factors influencing osseointegration of dental implants**

There are a lot of factors that influence osseointegration, amongst which systemic factors are considered to play an integral role. The implant and its host bone are the two major interacting entities, and minor changes to either entity would affect the process of bone formation around an implant. It is recognised that compromised bone negatively affect osseointegration, while a micro-rough implant surface results in superior osseointegration compared with a smooth surface. Many diseases and conditions may also affect bone quality. These include disorders of bone mineral homeostasis or imbalance of bone remodelling diseases such as osteoporosis, diabetes mellitus, hyperparathyroidism, Cushing’s disease, Paget’s disease, and collagen disorders such as osteogenesis imperfecta, Marfan syndrome, and drugs affecting bone quality such as glucocorticoids, chemotherapeutic agents and bisphosphonates, among others. [16]. Of these diseases, osteoporosis is the most prevalent worldwide; it is estimated that over 200 million people worldwide have osteoporosis[17]. In Australia, the effects of this disease causes fractures in 50% of women and 33.33% of men.
beyond the age of 60 years [18]. Osteoporosis causes reduced bone formation, leading to fractures and impaired bone healing. The relationship between osteoporosis and bone formation around implants is still unclear. The consensus of the ITI (International Team of Oral Implantology) is that there is only a weak association with the risk of implant failure, however, this conclusion was based on only two-case control studies[19]. This aim of this project is to observe the influence of osteoporosis on osseointegration on micro-roughened and smooth implant surfaces.

1.2 Scope of the project

The prime focus of this project was to observe the effects of the two factors—osteoporosis and surface morphology on osseointegration in rat maxilla. In order to clarify it, we divided it into four parts. The first part was designed to evaluate bone quality in the posterior maxilla of ovariectomized rats in order to validate this site as an appropriate model to study the effect of osteoporotic changes. Micro CT and histological analysis were used to evaluate bone changes at different time points over a period from 8 to 20 weeks post-ovariectomy. The study established a method to quantify the changes of intra-radicular alveolar bone in the posterior maxilla in an accepted rat osteoporosis model. The second part assessed the suitability and feasibility of placing a dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model. The results showed the mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research. The third part was designed to visualize the ultrastructural of osseointegraton in vivo. The results suggested an important regulatory role for osteocytes and their lacunar–canalicular network in maintaining long term osseointegration. Based on the above research, the fourth part was firstly to observe whether estrogen deficiency would affect the implant healing in the posterior maxilla area as well as whether the commercial modified rough surface implant can overcome the negative influence of osteoporosis.
1.3 Thesis outline

1 Introduction of thesis

2 Hypothesis & Aims: Based on the total hypothesis and aims of this project, which was further divided into four small projects. Following is a brief overview of the contents about the project.

3 Literature Review: The literature review is divided into two parts.
   - The first part was about the bone environment and the compromised factors on osseointegraton.
   - The second part reviewed the possible relationship between the osteocyte and dental implant.
     (Manuscript in preparation: “The emerging roles of osteocytes in osseointegration”)

4 First experimental chapter: The rat maxilla model of osteoporosis. This chapter answered the question about whether estrogen deficiency would affect the rat maxilla.
   (Paper accepted: “Estrogen Deficiency-Associated Bone Loss in the Maxilla: A Methodology to Quantify the Changes in the Maxillary Intra-radicular Alveolar Bone in an Ovariectomized Rat Osteoporosis Model”).

5 Second experimental chapter: The second part assessed the suitability and feasibility of placing dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model.
   (Paper accepted: "Evaluation of the 1st maxillary molar post-extraction socket as a model for dental implant osseointegration research”).

6 Third experimental chapter: to visualize the ultrastructural of osseointegraton in vivo and explore the possible role for osteocytes and their lacunar–canalicular network in osseointegration.
   (Paper accepted: "The Ultrastructural Relationship Between Osteocytes and Dental Implants Following Osseointegration.").

7 Fourth experimental chapter: This chapter addressed the questions of whether estrogen deficiency would affect the implant healing in the posterior maxilla area as well as
whether a modified rough surface implant could overcome the negative influence of osteoporosis.

(Manuscript in preparation: “The effects of osteoporosis and implant topography on osseointegration in rat maxilla.”).

8 Concluding Remarks: Summary of key findings and conclusions of the project.

**Ethics approvals for the PhD project**

Part of samples (the first and third part of the research) come from China, research protocols were approved by the Animal Care and Use Committee of Fujian Medical University (181/2009).

The other experimental protocols was proved by The Griffith University Animal Ethics Committee (DOH/01/4/AEC).
1.4 References


Chapter 2: Hypothesis and Aims
2.1 Hypothesis

Osteoporosis is one of the widespread bone disorders characterized by decreased bone mineral density and a deterioration of bone microarchitecture and deserves further discussion. Titanium dental implants have been established as a predictable treatment modality for tooth replacement, and micro-rough surfaced implants have been used to improve osseointegration. However, the extent to which osseointegration is affected by osteoporosis is yet to be fully elucidated, and it is yet to be determined whether micro-scale modified implants can overcome the negative influence of osteoporosis. The hypotheses of this project were: 1) Estrogen deficiency induced by ovariectomy leads to reduced bone density in the rat maxilla; 2) Estrogen deficiency would impair the osseointegration process of dental implants placed in the dental maxilla; 3) Moderately rough surfaced implant can improve osseointegration in this compromised bone environment; 4) Osteocyte play an important role in the process of osseointegration.

2.2 AIMS OF THE PROJECT

AIM 1: To verify that the rat maxilla undergoes changes following ovariectomy induced estrogen deficiency, and hence represents an appropriate model for osteoporosis research.

AIM 2: To verify that the rat maxilla is an appropriate model for dental implant osseointegration research.

AIM 3: To observe the ultrastructural aspects of bone-implant interface following osseointegration, especially the relationship of osteocytes with the dental implant.

AIM 4: To observe the effects implant surface topography of on implant osseointegration in the rat maxilla under estrogen deficiency induced osteoporotic-like conditions.
The study was divided into four parts.

Part 1  Rat maxilla osteoporosis model study

The effects of estrogen deficiency on bone characteristics are site-dependent, with the most commonly studied sites being appendicular long bones (proximal femur and tibia) and axial bones (vertebra). The effect on the maxillary and mandibular bones is still inconsistent and requires further investigation. This study was designed to evaluate bone quality in the posterior maxilla of ovariectomized rats. The design as follows:

Rats were randomly divided into two groups: an ovariectomized group (OVX, n=24) and Sham-operated group (SHAM, n=24). Six rats were randomly sacrificed from both groups at time points 8, 12, 16 and 20 weeks. The samples from tibia and maxilla were collected for Micro CT and histological analysis.

The volume of interest (VOI) region focused on the furcation areas of the first molar and second molars of maxilla. Initially, a circle with 40 pixel (0.64 mm) diameter was selected at the most coronal aspect of the root furcation, and after a further 40 slices apically a circle was selected with 60 pixel (0.96 mm) diameter. A third circle with the same diameter was then selected after another 60 slices and eventually, the VOI was defined by morphing across the slices between these three circles, forming an irregular, conical cylinder.

Trabecular bone volume fraction (BV/TV, %), trabecular thicknesses (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and connectivity density (Conn.Dens) were determined for VOI using the micro CT evaluation software SCANCO (Scanco Medical AG, Bassersdorf, Switzerland) provided with the micro-CT system.

Part 2  Rat maxilla implant model study

Published information regarding the use of rat jawbones for dental implant osseointegration research is limited and often inconsistent. This study assessed the suitability and feasibility of placing dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model. The design as follows:
Titanium implants (2×3mm) were placed bilaterally in the maxillary first molar area of 21 Sprague-Dawley rats. The animals were subsequently sacrificed at days 3, 7, 14, 28 and 56 post-surgery. Samples were prepared for histological and histomorphometric analyses.

Histomorphometric analysis of each specimen was then determined using ImageScope software (Aperio Technologies Inc). The percentage bone implant contact (%BIC) and the bone area (BA) were measured and analyzed.

**Part 3 The ultrastructural of osteocytes with implants**

Osteocytes influence bone remodeling by controlling the differentiation and activity of osteoblasts and osteoclasts. Determining the relationship(s) between titanium implants and osteocytes may therefore benefit our understanding of the process of osseointegration. The study was designed to observe the ultrastructure relationship of osteocytes with implant. The design as follows:

Titanium implants were placed in the maxillary molar region of eight female Sprague Dawley rats. The animals were sacrificed 8 weeks after implantation and undecalcified tissue sections were prepared. Resin-cast samples were subsequently acid-etched with 37% phosphoric acid prior to examination using scanning electron microscopy (SEM).

The sections were coated with gold and examined using a Carl Zeiss SIGMA VP field emission scanning microscope (FE-SEM; Carl Zeiss. SMT GmBH, Oberkochen, Germany) operated at a voltage of 15 or 20 kV. The focus of the ultrastructural analysis was the interface between bone and the implant surface. Elemental analysis was performed using the polished samples coated with gold with the same scanning microscope.

The morphology of osteocytes nearly implants were observed and analyzed.

**Part 4 The effects of osteoporosis and implant topography on osseointegration in the rat maxilla.**

Whether osteoporosis would affect osseointegration is still a controversial issue. Furthermore, researchers rarely use osteoporotic animal maxilla models as a research object to evaluate whether it is a risk factor for dental implant healing. Empirical study proves rough
surface titanium implants would result in superior bone to implant contact compared to traditional machined (namely smooth surface) implants. Whether the rough surfaced implant could reduce the osteoporotic influence on the implant healing process deserve study. The relationship of estrogen deficiency — osseointegration is still not clear. This study was designed to observe the effects of osteoporosis and implant morphology on osseointegration in rat’s maxilla, while the possible role of osteocytes will also be investigated. The design as follows:

Moderate rough surface implant and smooth surface implant were immediately implanted into the first molar mesial root socket separately after tooth extraction from the maxilla of both ovariectomy (OVX) and sham-operated (SHAM) rats. The rats were sacrificed at 3, 7, 14, 28 days separately for the observation.

Bone healing process and osseointegration were evaluated through histological observation, cryo-fracture plug SEM observation, and real-time PCR.

The expression of targeted genes, including alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen(COL1A), Receptor activator of nuclear factor kappa-B ligand (RANKL), Tartrate-resistant acid phosphatase (TRAP), Dentin matrix protein 1 (DMP1), sclerostin (SOST), Tumor necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β) were analysed.
Chapter 3: Literature Review
3.1 Review 1

3.1.1 The biology of bone tissue

Bone is the main constituent of the skeletal system, which primarily functions as the supporting framework of the human body. Aside from this structural function, it also serves many other purposes such as protecting the various organs of the body, assisting mobility, formation of hematopoietic cells and storage of minerals (especially calcium and phosphate).

The total number of bones in the human body is 206, and they are categorized into different types based on their organization: the axial skeleton and appendicular skeleton. From an anatomical perspective, bone also can be defined as: flat bones (skull bones, mandible, scapula and ilium) and the long bones (limb bones – humerus, ulna, radius, femur, tibia, fibula etc.). Bone is composed mainly of mineralized osseous tissue. Besides its cellular components (i.e. the osteoblasts and osteocytes), the bone tissue includes marrow, endosteum and periosteum, nerves, blood vessels and cartilage. The origin of bone formation during the fetal stage of development occurs by two processes: intramembranous ossification and endochondral ossification. Intramembranous ossification is responsible for formation of most of the flat bones of the skull but also the mandible, maxilla, and clavicles, and osteogenesis occurs directly and without any intermediate cartilage. The steps mainly include: development of an ossification centre, calcification, formation of trabeculae and development of periosteum. However, in endochondral ossification, (typically involved in the formation of the axial and appendicular skeletons), osteogenesis occurs directly from mesenchymal cells initially condensing to form a cartilage intermediate, which is eventually replaced by mineralized tissue. The steps may be summarized as follows: Development of cartilage element, growth of cartilage element, development of the primary ossification center, development of the secondary ossification center, formation of articular cartilage and epiphyseal plate.

Bone tissue classification is based on histological appearance as either compact (cortical bone) or spongy (trabecular/spongy bone). Cortical bone is important for providing the
supporting structure and surrounds the trabecular bone. Spongy bone on the other hand, contains inter-connected bony trabeculae. Based on the mineral arrangement, bone also can be divided into lamellar bone and woven bone. Lamellar bone characteristically has well-structured morphology, consisting of concentrically (in compact bones) or parallel (in spongy bones) organized lamellae of collagen fibers. In compact bones, the concentrically arranged lamellae surround a central channel that contains blood vessels and nerve fibers (Haversian canal). Another system of canals (Volkmann’s canals) run perpendicular to the Haversian system and provide inter-connection between the inner and outer layers of bone. Woven bone is immature and does not have any orderly arrangement of collagen fibers as is the case with lamellar bones. Such bone is formed in regions requiring rapid bone formation, for example, fracture healing.

Bone tissue consists of a mineral phase intimately embedded into an organic matrix[1]. The mainly inorganic phase of bone is hydroxyapatite, a calcium phosphate apatite. The Ca/P ratio of bone tissue varies from 1.50 to 1.90, depending on age and the bone site. However the ideal atomic ratio of Ca/P is 1.67, but this may be affected by aging or bone disease[2].

3.1.2 Bone cells

There are three different types of cells – **osteoblasts**, **osteocytes** and **osteoclasts** existing as the essential living components of bone tissue. Osteoblasts originate from mesenchymal stem cells and are located on the surface of osteoid seams. They make a protein matrix known as osteoid that is mineralized to form bone. Some of the terminally differentiated osteoblast cells eventually get trapped in the lacunae of the matrix and form osteocytes. Osteocytes embedded in bone tissue are the most abundant cell population, comprising ~90-95% of all bone cells in adult bone and are the longest lived bone cell [3]. The osteocytes are distributed throughout the bone matrix and are interconnected to one another, as well as cells on the surface and the bone marrow through their extensive dendritic network. They form an extended, three-dimensional (3D) network, with processes interconnecting the cell bodies residing in thin canals, known as canaliculi. Together with the osteocyte lacunae, the
canaliculi form the lacuno-canicular network (LCN) [4]. (Fig.1) They have recently been found to function as an orchestrator, regulating both osteoclast and osteoblast activity and mineral metabolism[5]. The third type of bone cell is the osteoclast, and osteoclasts comprise 1-2% of bone cells. They are formed by the fusion of mononuclear cells of the monocyte/macrophage lineage. Osteoclasts are polarized multi-nucleated cells which are responsible for bone resorption in the bone remodelling process. It is believed that the monocyte/macrophage colony–stimulating factor (M-CSF) and the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) are the essential factors for differentiation of osteoclasts [6, 7]. The interplay between osteoblasts and osteoclasts is responsible for maintaining normal bone homeostasis in the human body. Osteocytes modulate the activity of both of these types of cell. Mature osteocytes are viable for about 10 years, osteoblasts for about 9 months and osteoclasts for about 3 weeks[8].

As the most important cell in creating and maintaining skeletal architecture, osteoblasts are derived through the differentiation of osteogenic cells in the periosteum (tissue that covers the outer surface of bone), and in the endosteum of the marrow cavity (usually located in the deeper layers of the periosteum and bone marrow). Osteoblasts are the principal cells in the bone forming process. During the process of osteoblast differentiation, osteoprogenitor
cells have the ability to express Runx2/Cbfa1 transcription factor, which regulates the expression of several osteoblastic genes, such as COL1A1, alkaline phosphatase (ALP), bone sialoprotein (IBSP), SPP1 (osteopontin) and BGLAP (osteocalcin)[9]. Other osteogenic markers that are expressed during differentiation include Osterix, M-CSF, osteocalcin (OC) and osteonectin (ON). Once the bone has formed, most of the mature osteoblasts undergo apoptosis, while a few of them transform into the bone lining cells known as osteocytes. (Fig.2, 3)

![Fig.2 Expression of markers during osteoblast-to-osteocyte ontogeny][5]

![Fig.3 Origin of osteoblasts in humans. The osteoblast precursors proliferate and give rise to osteoblasts which in turn lay the bone matrix and also give rise to the osteocytes][10]

Morphologically, mature osteoblasts are cuboidal in shape, with their size varying between 20-30µm in diameter[11]. Osteoblasts become flattened and elongated upon
maturation. These cells have cytoplasmic projections that allow them to form communications with adjacent osteoblasts and osteocytes. When they are active, a large Golgi apparatus and an abundant rough endoplasmic reticulum is visible[12]. Osteoblasts usually form a single layer of cells on the surface of bone. However in cases where there is active bone formation, they may be present in the form of layers[11].

The principal function of osteoblasts is bone formation. As mentioned earlier, these cells are responsible for the deposition of bone matrix and for osteoclast regulation, and when they differentiate they obtain the ability to secrete bone matrix [13]. They are also responsible for the synthesis of various kinds of proteins and polysaccharides. These cells also play an important role in bone remodelling by maintaining a balance between bone formation and resorption during new bone formation. Rarely, osteoblasts are also known to initiate the bone resorption process. Mature osteoblasts synthesize several phenotypic markers like, type I collagen, alkaline phosphatase (ALP) and osteocalcin (OC).

3.1.3 Bone remodelling cycle and the key role of osteocytes

The bone remodelling process can be defined as the removal of mineralized bone by osteoclasts, followed by the formation of a bone matrix by osteoblasts that subsequently become mineralized. The continual process of adult bone remodelling is important for the maintenance of bone mass and skeletal microarchitecture [14]. Osteocytes, osteoclasts and osteoblasts are the basic multi-cellular unit of bone remodelling (BMU) which is covered by a ‘canopy’ of cells (most probably bone-lining cells) that together form the bone remodelling compartment (BRC) [15] (Fig.4). There is increasing evidence that osteocytes play an important role in the cycle of targeted bone remodelling. In this context, it is noteworthy that the entire bone surface is not constantly undergoing active remodelling. Indeed, over 95% of the surface of the bone tissue is inactive because of osteocytes exerting a quiescent inhibition of both osteoclastic bone resorption and osteoblastic bone formation [16]. By contrast, once local skeletal microdamage occurs or the mechanical loading decreases, osteocytes react either by secreting cytokines and chemoattractants or by undergoing
apoptosis. These reactions trigger local recruitment of osteoclast precursor cells and mature osteoclasts to initiate bone resorption [14]. Osteoclast resorption activity lasts over a period of 3–5 weeks until this process is terminated by apoptosis and followed by recruitment of osteoblast precursors. Subsequently, in the next 3 months osteoblasts secrete and mineralize osteoid to replace the resorbed bone [16]. The whole process is regulated by osteocytes, which alter the rate of bone remodelling by controlling osteoclast formation via the production of RANKL. On the other hand, osteocytes also control the balance between formation and resorption by regulating osteoblast formation via production of sclerostin, which is encoded by the SOST gene and highly expressed in mature osteocytes. Its primary function is to impede bone formation. Sclerostin may have additional functions, such as stimulation of RANKL expression by osteocytes [15, 17]. The balance between osteoclast and osteoblast activity results in skeletal homeostasis with preservation of bone strength[18]. In brief, the bone remodelling cycle is initiated and orchestrated by osteocytes, and propagated through coupled cross-talk between osteoblasts and osteoclasts.

![Fig.4 The bone remodelling compartment (BRC) comprises the cells constituting the basic multi-cellular unit (BMU)](15)
3.1.4 Disorders of bone homeostasis

One of the important functions of bone is the housing and support of hematopoiesis, any reason causes the bone homeostasis may possibly damage its function. The above described bone remodelling process is a cycle of resorption, carried out by osteoclasts, and formation by osteoblasts. In the adult skeleton, an appropriate balance between the processes of bone formation and resorption is necessary for maintenance of appropriate bone homeostasis. The balance between bone formation and resorption is also vital to avoid microdamage accumulation and to preserve the proper material and structural integrity of the bone. This balance may be disrupted in certain situations leading to clinical conditions causing significant morbidity. While individual conditions may have a genetic and/or developmental etiology (e.g. osteogenesis imperfecta); others are associated with the cumulative effects of predisposing factors (e.g. osteoporosis). Some of the common disorders of bone homeostasis are osteoporosis, rickets and osteomalacia, Paget’s disease of bone, renal osteodystrophy and osteogenesis imperfecta. Osteoporosis is a widespread bone disorder characterised by decreased bone mineral density and a deterioration of bone microarchitecture. Therefore, the issue of whether osseointegration would be affected by osteoporosis is clinically relevant and warrants ongoing investigation.

3.1.5 Osseointegration

When an implant is placed into bone tissue, the healing process is very important for future implant success. Although many studies have tried to investigate the mechanisms of osseointegration, the regulatory factors of bone formation around implants are still not fully understood. Bone healing around implants is considered similar to the fracture bone healing process wherein blood is invariably the first tissue that the implant will contact. This contact results in the activation of a cascade of biological processes in the gap between the implant and the bone tissue, such as protein deposition, coagulation, inflammation, and tissue formation[19]. Animal studies have revealed that when implants are put into the bone cavity, the gap between the implant and bone tissue is immediately and rapidly filled with blood clot,
red blood cells, inflammatory cells mainly consisting of neutrophils, and degenerating cellular elements[20, 21]. After 3-5 days, a layer of flattened cells with interposed blood capillaries lines the implant surface and the intercellular spaces are filled with fibrin. In 5–7 days after implantation, the newly formed bone tissue/osteoid mixed with cuboidal cells can be observed on the implant surface [22]. Meyer et al reported that just 3 days after implantation some small bone-like crystals can be observed at the implant surface [23]. At 28 days after placement, the implant appears well osseointegrated [22, 24]. Newly formed bone can be seen around the implant surface forming a reticular structure with wide marrow spaces at trabecular areas [24]. A primary spongiosa, rich in vascular structures is also reported [21]. Although many researchers have histologically described the process of osseointegration, the cellular and molecular events in the early bone formation around implants have not been clarified. Different cell types, growth factors and cytokines are all involved in a co-ordinated manner during the inflammatory, formation and remodelling phases of bone healing. This means that osseointegration should be regarded not as an exclusive reaction to a specific implant material, but as an endogenous bone regenerative process [25].

Until now, knowledge of "osseointegration" of titanium implants is mainly based on clinical experience and light microscopic observations (LM) [26]. In order to promote osseointegration around bone tissue, researchers need to know the exact response at the interface, which includes the material and host response. As we know, the material response consists predominantly of oxidation of the metallic implant surface, which undergoes a string of electrochemical changes in the physiological environment. The material selected for implants must have a stable oxide film, and for the commercially pure Ti implants routinely used in dental implantology, the thickness is 2-6nm before implantation [27]. The design, chemical composition and topography of the implant surface can also influence peri-implant tissue healing [28]. Rough surfaces have been shown to promote osseointegration [29, 30].

After implantation, the host response is the main determinant of the wound healing response in the early stages of the osseointegration process. The early response after implantation into bone tissue is the adsorption of proteins, which subsequently undergo
desorption (native or denatured, intact or fragmented) or remain to mediate the subsequent tissue-implant reaction [31, 32], including cell adhesion and binding to the material surface [33, 34]. The interaction of red blood cells, fibrin and platelets with the implant surface may modulate the migration, differentiation and activity of osteogenic cells during peri-implant healing [35, 36]. These events are the early biological processes and osteoblasts have been found to attach on the implant surface from day one of implant insertion. [23]. The attached osteoblasts start depositing collagen matrix directly on the implant surface, forming a cement line/lamina limitans layer, which is an early-formed calcified afibrillar (0.5mm thickness) layer containing abundant amounts of calcium, phosphorus, osteopontin and bone sialoprotein [37]. However, whether osteoblasts actually interact directly with the implant surfaces in vivo is still controversial [38].

Bone tissue is constantly metabolised throughout life. These metabolic activities are executed by bone-resorbing osteoclasts and bone-forming osteoblasts, cells that are recruited from the bony environment. Mounting evidence suggests that osteocytes can modulate this process [39-41] and play an active role in the bone metabolic process through the lacuno-canalicular system [5]. Therefore, elucidation of the role of osteocytes in the remodelling of the bone surrounding implants will help to understand the dynamic bone metabolic process following osseointegration. Since osteocytes are the terminal cells of osteoblasts, understanding the relationship of osteocytes with implants also benefit comprehension of early bone formation relationship between osteoblasts and implants.

3.1.6 Osseointegration Under Compromised Conditions

Dental implants manufactured from titanium have become a well-established treatment modality for the replacement of missing teeth. The clinical procedures using dental implants are well documented, with good long term success rates reported in healthy patients and in favourable anatomical positions [42]. However, with increased clinical use, greater acceptance and popularity of implants, there are greater demands on implant systems from both clinicians and patients. In particular, there is demand for implant placement in sites
where the quality of bone is less than ideal, such as those encountered in systemic conditions where the amount of mineralised tissue is reduced (osteoporosis) and/or the bone wound healing is compromised (uncontrolled diabetes mellitus or the use of intravenous bisphosphonates).

Due to the increasing clinical demands, there are continuing efforts to enhance the rate and amount of osseointegration, especially in compromised scenarios. One of the parameters which have been shown to influence the success rate of implants is the alteration of the surface topography by increasing the roughness of the implant surface [43]. Indeed, the use of microscale modifications of implant surfaces has been credited with being one of the key factors in increasing the clinical success rate of implants, especially in areas of compromised bone quality [44]. However, the cellular and molecular mechanisms responsible for these superior clinical outcomes are poorly understood [43]. Further improvements in implant design, surface characteristics and surgical protocol are hampered by a lack of understanding of the fundamental biological mechanisms that result in osseointegration, and in particular how these mechanisms are influenced by surface modification.

3.1.7 Compromised Clinical Scenarios

3.1.7.1 Osteoporosis

Osteoporosis (OP) is a bone-weakening systemic disease. It is estimated that over 200 million people suffer from this disease and over 40% of postmenopausal women experience a fractured bone some time during the rest of their lives[45, 46]. Osteoporosis is a condition characterised by decreased bone mineral density and a deterioration of bone microarchitecture, and hence compromised bone quality. It is prevalent in females and its incidence increases with age. In Australia, it is estimated that 50% of the women and 33.33% of men beyond the age of 60 years develop fractures due to osteoporosis [47]. Osteoporosis can be divided into primary and secondary types. The primary type also can be sub-divided into type I and type II [48]. Our research is mainly focused on the type I that is Post-
menopausal (type I) osteoporosis, which is a common bone disorder in postmenopausal women and is caused primarily by estrogen deficiency resulting from menopause. This form of osteoporosis is exemplified by accelerated bone loss. The skeletal loss occurs primarily from trabecular bone, leading to distal forearm and vertebral body fractures. Type II is senile osteoporosis, and mainly occurs in both men and women over the age of 70 years and is due to the loss of cortical and trabecular bone. Fractures of the wrist, spine and hip are often seen with type II osteoporosis. Secondary causes of osteoporosis are due to disorders classified as genetic (congenital), endocrine, hypogonadal states, deficiency states, drug-induced, inflammatory states, hematologic, neoplastic and miscellaneous. (Fig.5)

Fig. 5. Scanning electron micrograph of normal bone (left) and osteoporotic bone(right). [49]

Estrogen deficiency is thought to be the critical in the pathogenesis of post-menopausal osteoporosis. In the post-menopausal period, due to estrogen deficiency, bone structure is reduced and changed. The studies have proved that both bone resorption and bone formation are increased in postmenopausal osteoporosis; however, the extent of increased bone resorption exceeds that of augmented bone formation, which causes an imbalance between bone resorption and bone formation in favour of bone resorption[50]. The understanding of the molecular and cellular mechanisms underlying the role of estrogen deficiency in the pathogenesis of postmenopausal osteoporosis was studied mainly in the 1980s and 1990s. In 1980s, research focused on the cytokines including interleukin (IL)-1, −6, and −7; tumour necrosis factor (TNF); granulocyte/macrophage colony–stimulating factor (M-CSF). The
breakthrough discovery was made in the 1990s, through the understanding of RANK/RANKL and OPG roles in osteoclastogenesis[50]. (Fig.6)

![Fig.6 Current understanding of the pathological mechanisms of postmenopausal osteoporosis. Dates indicate the time of the discovery of the functions. Abbreviations: FSH, follicle-stimulating hormone; IL, interleukin; M-CSF, monocyte/macrophage colony-stimulating factor; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; TNF, tumor necrosis factor[50].]

**Estrogen can directly and/or indirectly have effects on osteoclast, osteoblast and osteocytes.**

It is now firmly established that osteoblasts, osteocytes, and osteoclasts express functional estrogen receptors (ERs) [51]. Recent studies have clearly demonstrated that estrogen can induce osteoclast apoptosis, through osteoclast-specific deletion of ERα (using Cre driven from cathepsin-K or lysM, respectively), can reduce osteoclast apoptosis and relatively increase osteoclast lifespan which results in decreased trabecular bone mass[15, 52]. Estrogen also can induce ERα binding to a scaffolding protein, Breast cancer anti-estrogen resistance protein 1 (BCAR1); the ERα/BCAR1 complex then isolates TNF receptor-associated factor 6 (TRAF6), inhibiting RANKL-stimulated osteoclastic differentiation of human monocytes and leading to decreased activation of NF-κB and impaired RANKL-
induced osteoclastogenesis [53]. Estrogen also can inhibit RANKL-induced osteoclastogenesis through reduction of c-Jun activity (estrogen reduces c-Jun expression and decreases phosphorylation) to block RANKL/M-CSF-induced activator protein-1-dependent transcription. Estrogen can also indirectly regulate osteoclast formation through suppressing RANKL production by osteoblasts, T and B cells [54] and also increasing production of the decoy receptor for RANKL, osteoprotegerin (OPG) [15, 55]. Estrogen can direct effects on osteoblasts through three pathways: inhibiting apoptosis, suppressing oxidative stress, and decreasing NF-kB activity. The effects of estrogen on reducing osteoblast apoptosis are due to activation of the Src/Shc/ERK signalling pathway. Aging and estrogen deficiency may lead to impairment in bone formation involving oxidative stress. Estrogen deficiency also can lead to a marked increase in NF-kB activity in osteoblast cells. Using estrogen treatment, osteoporosis patients would induce the potent inhibitor of bone formation, sclerostin[56]. As sclerostin is expressed by osteocytes, this in turn means estrogen also affects osteocytes. (Fig.7)

![Fig.7 Working model for estrogen regulation of bone turnover via effects on osteocytes, osteoblasts, osteoclasts, and T cells [15].](image)

The effects of estrogen on jaw bone compared with long bone has been a controversial issue until now[57]. The reasonable explanations for the inconsistent reactions of jaw bone
compared with long bone were always attributed to the differences of ossification patterns and embryological origins. Jaw bone is both morphologically and functionally different from the other bones of the axial or peripheral skeleton. It also derives from a different embryonic origin (neuroectoderm) compared with bone of the axial and appendicular skeleton, which arises from mesoderm[58], and the intramembranous ossification of jaw bone differentiates with the endochondral ossification of long bone. From recent research, it appears that the differences of long bone and jaw bone reaction to estrogen deficiency are based on inherent genetic differences between the bone of cranial origin and the bone of non-cranial origin[59]. This seems to explain the behaviour differences of osteoblasts between jaw bone and long bone[60]. Following the development of dental science, especially as dental implants become a mainstream restoration for the edentulous patients, dentists are more careful about the effects of systematic disease on jaw bone. It has been hypothesised that osteoporosis affects the jaws in the same manner as other bones of the skeleton, and thus may also alter bone metabolic microenvironments around the implant. Since it is well recognised that dental implants, especially those with a machined surface, perform worse in areas of poor bone quality such as the posterior maxilla, osteoporosis can be considered a good model for the study of osseointegration in poor quality bone. Furthermore, osteoporosis itself has been shown to be a risk factor for implant failure [61], although it is unclear if this negative effect is overcome by the use of implants with a micro-rough surface topography [62]. Determining the precise extent to which surface modification can influence healing in a poor quality bone environment through the use of an osteoporotic model has important clinical implications, especially given the extensive incidence of osteoporosis in the general population.

Of all the animal models of osteoporosis, the ovariectomized rat has been selected as the preferred model to simulate human post-menopausal osteoporosis. This is because both share many similar characteristics, including an increasment of bone turnover rate which represent by the resorption exceeding formation, an initial rapid phase of bone loss followed by a much slower phase; greater loss of cancellous bone than cortical bone; and similar skeletal response to therapy with medicines [63]. Other important reasons are ease of handling and housing,
[64]. However, because the jaw bone is small and difficult to operate on, the potential for using jaw bone as a research object for implants is limited, especially the maxilla. Additionally, the potential for using a rat’s maxilla is limited compared to the mandible. The reason presumably being that the anatomy of maxilla is more complicated compared to the lower jaw.

In brief, whether the jaw bone is affected by osteoporosis is still a controversial issue, the prerequisite condition for using the rat maxilla as an osteoporosis model to observe the bone-implant healing process, is to first identify whether this site has become osteoporotic.

3.1.7.2 Microscale titanium surface modification and osseointegration

The design, chemical composition and topography of the implant surface can influence peri-implant tissue healing [28]. Commercially pure titanium (Ti) is widely used as a dental and orthopaedic metallic implant material. It is a highly biocompatible material with good resistance to corrosion, no toxicity on macrophages or fibroblasts, and lack of inflammatory response in peri-implant tissues. However, smooth or machined implant surfaces have been replaced by topographically modified microroughened implants in recent years. Animal studies have shown that micro-rough titanium implants result in superior bone to implant contact compared to machined (minimally rough) implants [65, 66], as well as having superior torque removal values [67, 68]. Histological analysis of the sequential healing events associated with the placement of micro-rough surface titanium implants shows that initial bone formation around these implants occurs not only at the exposed bone wall of the surgically created implant recipient site, as is the case with machined surface implants, but also along an “osteophilic” implant surface [21]. The study also demonstrated histological evidence of superior early healing is associated with the micro-rough surfaces, which shows a higher level of organization in the wound observed as early as 4 days following implantation, ultimately leading to greater bone-implant contact one week post-insertion. These findings suggest that key biological events leading to the superior histomorphometric characteristics and clinical performance of modified implant surfaces occur early in the wound healing
process at the tissue-implant interface. Thus, during osseointegration, implant surface topography appears to influence cellular and molecular mechanisms at the tissue-implant interface.

Given that implant therapy has a very high success rate in healthy patients with good bone quality and quantity, one of the major foci of current research is the performance of implants in compromised sites and patients. Therefore, understanding the extent to which microscale modified implants can overcome the negative influence of systemic bone conditions has a clear clinical relevance.

3.1.7.3 Biological mechanisms

Osseointegration is a complex process that involves a cascade of events that occur at the tissue-implant interface. These involve clot formation and the initial adsorption of serum components immediately following implant placement, an immune-inflammatory response to implant insertion, the migration and attachment of undifferentiated mesenchymal cells on the implant surface, their proliferation and differentiation, and finally, the formation of extracellular matrix and its mineralization and maturation. However, little is known about the mechanisms which are affected by systemic disease and how they may be modulated by surface modification. Recent studies have shown that using a human *in vivo* model inflammation, the main biological processes that are involved in osseointegration are skeletogenesis, angiogenesis and neurogenesis. [69, 70]. Concurrently, IkB/ NFkB, Wnt and TGfβ/BMP signalling is prominently regulated during the osseointegration process. Although the biological processes of osseointegration are now beginning to be elucidated, little is known about the mechanisms via which systemic disease can influence osseointegration, or how titanium surface micro-roughness can modulate this effect.
3.2 Review 2 The emerging roles of osteocytes in osseointegration

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(Manuscript prepared for Clinical Oral Implants Research)

3.2.1 Abstract

The recent revelation that osteocytes play a crucial role in the maintenance of bone homeostasis has challenged the conventional view that they are passive placeholder cells. As mechanosensors, osteocytes sense mechanical strain and are known to orchestrate the activities of osteoblasts and osteoclasts and are therefore seen to play several key regulatory roles in bone modelling and remodelling process. Osseointegration is a dynamic process that is thought to include all the activities similar to bone remodelling process as it occurs in the natural bone environment. This review seeks to reconcile the gap between the existing knowledge of osteocytes and osseointegration, explore possible mechanisms of osseointegration, and highlight the important role of osteocytes in the osseointegration process, a role which is worthy of further investigation.

Key words: Osteocyte, Osseointegration, Implant, Bone, Remodel
3.3.2 Background

When Brånemark, the "father" of modern dental implantology, introduced the concept of osseointegration, it heralded a revolution in the field of dental implantology and established metallic implants as one of the mainstream rehabilitation modalities for dental reconstruction procedures [71, 72]. Conventionally, osseointegration has been considered as the favourable outcome of the bone tissue healing process around an implant surface and as such is considered to be the key biological process responsible for long term implant stability. Researchers are constantly trying to improve implant materials in order to achieve a superior osseointegration. Although there are a number of studies that have tried to unravel the mechanisms of osseointegration, the factors regulating bone formation around implants are still not fully understood.

Bone healing around dental implants is similar to the fracture healing process in many ways. Clotted blood is invariably the first tissue to come in contact with the implant. This leads to the activation of a cascade of biological processes in the gap between the implant and the bone tissue, such as protein deposition, coagulation, inflammation, and tissue formation [19]. A comprehensive temporal course of molecular events following implant placement have been demonstrated in animal studies. The gap between the implant and bone tissue is immediately seen to be filled with blood clot, red blood cells and inflammatory cells (mainly consisting of neutrophils, and degenerating cellular elements) [20, 21]. A layer of flattened cells and some interspersed blood capillaries can be seen to line the implant surfaces after 3 to 5 days of implant placement and the intercellular spaces are filled with fibrin (Fig. 1) [73]. Newly formed bone tissue/osteoids, mixed with cuboidal cells, is observable on the implant surface within 5 to 7 days [22]. Within 28 days, the implant usually appears to be well integrated with the surrounding bone [22, 24] and newly formed bone with a reticular structure containing wide marrow spaces at trabecular areas is visible around the implant surface [24]. Although many studies have described the process of osseointegration from a histological perspective, the regulation of cellular and molecular events, especially in the early bone formation period around implants has not been well elucidated.
Fig. 1 Early stages of the healing process (day 4) on active SLA implant surface shows fibrin network filled the bone and implant space with many cell types including red blood cells, platelets and white blood cells.

The process of bone healing around implants, involves coordinated interactions between different cell types, growth factors and cytokines that modulate the inflammatory, bone formation and remodelling phases. Osseointegraion involves osteogenic differentiation of mesenchymally derived stem cells, which are transformed into cells of the osteogenic lineage: preosteoblasts, osteoblasts and osteocytes, and the coupled activities of osteoclasts and osteoblasts. This process, ultimately leads to the formation of the hierarchical mineralised bone tissue around the implant, maintaining its long-term “survival” in the oral cavity.

Research into osseointegration has tended to focus on the “pre” and “pro” osteogenic mechanisms such as the role of blood clot and its components or the activation of pro-osteogenic osteoblastic cellular mechanisms on implant surfaces. However, the influence of the predominant bone cell type—the osteocyte–has often been overlooked. Osteocytes have recently been found to play a leading role in the bone metabolic process– regulating the activities of both osteoblasts and osteoclasts. It is, therefore, plausible that osteocytes play a key role in the process osseointegration, something future research should be able to uncover [74].

In a recent study, we examined the morphological differences between tissue samples from well-osseointegrated (successful) and non-osseointegrated (unsuccessful) implants in rat
animal. Contrary to our expectation, majority of the unsuccessful samples showed no obvious signs of inflammation or bone resorption, nor were there any observable changes in the bone wall of the implant bed. There was, however, significant difference in osteocytes morphology at the implant-bed interface (“interface osteocytes”) between unsuccessful and successful implants. In the unsuccessful cases, the interface osteocytes had shrunken cell bodies and fewer dendrites, and many were dead. By contrast, in successful cases the interface-osteocytes were seen to have a normal phenotype in the form of an oval shaped cell body and many dendrites extending from the cell body (Fig. 2) [75]. These results are similar to a study in which bone formation was reduced following the targeted ablation of osteocytes using diphtheria toxin [76]. Results such as these support the hypothesis that osteocytes play an important role in orchestrating the process of osseointegration. In this review we attempt to interpret the existing literature on the functions of osteocytes in order to draw a picture of the regulatory role of osteocytes during osseointegration.

Fig. 2: Comparison of a successful vs failed dental implant. The successful implant (A and B) shows close contact and normal osteocytes on the implant surface; whereas the failed implant (C and D) shows an absence of osseointegration and osteocyte with changed morphology and the loss of dendrites (red arrows).

Bone is a complex and highly dynamic tissue organized in a hierarchical pattern containing both organic and inorganic components. The organic component consists of proteins, mainly type I collagen fibres, and bone cells. The inorganic component
predominantly consists of hydroxyapatite crystals. There are three major types of bone cells: osteoblasts, osteoclasts and osteocytes being the living components of bone tissue.

3.2.3 Relationship of osteoblasts with implant

Osteoblasts originate from mesenchymal stem cells, transforming through a preosteoblastic phase, and secreting mineralised bone tissue. There are three destinies for osteoblasts: firstly, some of the osteoblasts become apoptotic osteoblasts, secondly, become into bone lining cells, and the third is some osteoblasts get embedded in the mineralised matrix and form terminally differentiated osteocytes[77]

Conventional wisdom holds that osteoblasts play a leading role in the bone healing process around implant, which has been investigated extensively both in vitro and in vivo [23, 78-86]. However, in vivo experiments have yet to conclusively demonstrate if osteoblasts actually interact directly with the implants [38]. It is generally thought that mesenchymal stromal cells (MSCs) within the bone environment will eventually differentiate into osteogenic cells following the placement of an implant. Within the first day of an implantation MSCs and osteoblasts-like cells can be found around the implant surface, where they deposit bone-related proteins and create an environment conducive for cell adhesion and mineralization [87, 88]. During the healing process, osteoprogenitor cells express RUNX2/Cbfa1 transcription factor that regulates the expression of several osteoblastic genes, such as type I collagen (COL1), alkaline phosphatase (ALP), bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN) [9]. Osteoblasts secrete all of these factors; they are therefore widely used for analysis of the activities of osteoblasts around dental implants [89-92]. The cells attain the ability to secrete bone matrix upon differentiation [13] and are responsible for the synthesis of a number of proteins and polysaccharides. The secreted protein matrix, known as the osteoid, entraps some of the mature osteoblasts, which prompts their transformation into osteocytes and subsequent mineralization around the implant. Recently the view that osteogenic differentiation of MSCs is regulated by osteoblast [93, 94] has been challenged by studies that suggest that it is osteocytes that have the lead role in regulating the differentiation of MSCs [95-97]. However, the exact molecular mechanisms
involved in guiding the transition of osteoblasts to mature osteocytes in this context are still elusive [77, 98].

### 3.2.4 Relationship of osteoclasts with implant

Osteoclasts are cells derived from the hematopoietic lineage, which also give rise to monocytes and macrophages [99]. During the bone remodelling osteoclasts are primarily implicated in bone resorption. The regulatory role of osteoclasts in osseointegration process is not well understood but osteoclasts are known to participate in the bone remodeling process around implants as early as day 7 following placement, and have an important role in the long term bone remodeling process [100]. It is believed that moderately rough implant surface can not only promote bone healing around implant, but also affect the proliferation and differentiation of osteoclasts in vitro [101-103]. Certain factors and pathways such as macrophage-colony stimulating factor (M-CSF), the receptor activator of NF-κB ligand (RANKL)-RANK pathway, and osteoprotegerin (OPG), an antagonist of RANKL function are known to play an essential roles for osteoclast differentiation and development [104, 105]. These factors have all been found in and around aseptic loosening of implants [106] or associated with bone remodling [107] and peri-implant tissue destruction [108, 109].

The process of bone remodelling is known to start with bone resorption caused by osteoclast; however, the recruitment and subsequent selection for differentiation at the site of prospective bone resorption remains unclear. The dynamic remodelling process involves osteoclast-guided bone resorption and the coupled osteoblast-guided bone formation. This process usually occurs in a basic multicellular units (BMUs) which include osteoclasts, osteoblasts, and osteocytes covered by a “canopy” of cells, possible formed by bone-lining cells [15]. The lining cells are believed to play a positive role in preparing and conditioning the sites of bone resorption [110]. Recently it has been reported that osteocytes play an active role in the recruitment of osteoclasts [98]. Osteocytes are to be the major source of RANKL during bone remodelling, effectively controlling osteoclasts formation [111, 112], and even have the potential to recruit osteoclasts to the sites of remodelling [98]. The osteocyte-like cell line MLO-Y4 are reported to express M-CSF, a decoy receptor of RANKL [113], which
suggest that osteocytes in the peri-implant healing compartment should be the subject of
further investigation.

3.2.5 The emerging role of osteocytes in osseointegration

Osteocytes embedded in bone tissue are the most abundant cell populations, comprising 90~95% of all bone cells in adult bone, and are the longest living cells in bone [3]. The conventional view is that osteocytes are passive placeholders; however, it is now realized that osteocytes have multi-functional roles such as a mechanosensor, and actively regulate bone modelling and remodelling process as well as having an endocrine function [74]. Osteocytes can sense mechanical strain and the developing microcracks through the loading-induced fluid flow which exist in the lacuno-canalicular network (Fig. 3) within the bone, via either the osteocyte body itself, its dendritic processes or the bending of cilia [114, 115], single flagella-like structures found on every cell [116]. Osteocytes also respond to hormonal changes (such as estrogen deficiency), by an increase of osteocytic apoptosis, which triggers bone remodelling and resorption [15]. Moreover, osteocytes can regulate of phosphate and biomineralization through the expressed molecules such as phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX), dentin matrix protein 1 (DMP-1), and fibroblast growth factor (FGF23) [117, 118]. Other routes through which osteocytes can affect bone remodelling is via Dickkopf-related protein 1 (DKK1) and Sclerostin (SOST), both of which are antagonists of the Wnt/β-catenin pathway, and highly expressed in osteocytes [15]. Therefore, it is now obvious that this “passive placeholder in bone” plays a far more important role in bone modelling and remodelling process than what has been previously thought. As osseointegration process involves mechanical stimuli (primary stability, masticatory force etc), bone modelling and remodelling interaction, the role of osteocytes in this process deserve more attention.
3.2.6 The important role of osteocytes in regulating osteoblast activities in osseointegration

If we appreciate the bone healing process as a result of cell proliferation and differentiation, then bone healing around the implant is an ordered process of cell differentiation, moving through the stages of pre-osteoblasts, osteoblasts, osteoid osteocytes, and mature osteocytes, with interposing osteoclasts activities at the same time. The end results is that the osteocyte dendrites make close contact with the implants—the process known as osseointegration [119]. Evidence shows that osteocytes are responsible for the osteogenic differentiation of MSCs [96]. There are numerous factors involved in this cell-to-cell process and demonstrating osteoinductive effects [120]. Using avian osteocytes [121] and the murine long bone osteocyte Y4 (MLO-Y4) cell line [122], it was shown that osteocytes release factors that regulate osteoblast differentiation and ALP activity [74, 121, 122]. Osteocytes may communicate with osteoblasts through gap junctional intercellular communication (GJIC), once they sense the mechanical stimuli, then transmit biophysical signals to osteoblasts [123]. Simultaneously they will secrete other factors such as prostaglandin E2 (PGE2), ATP, and nitric oxide (NO) (NO also inhibiting osteoclasts activity) to closely regulate osteoblasts behaviour [74]. Other secreted factors include insulin-like
growth factor 1 (IGF-1) [124, 125], osteopontin (OPN), and parts of bone morphogenetic proteins (BMPs) [126, 127]. PGE2 is a multifunctional regulator for bone metabolism and can increase the osteocyte gap junction function and plays an important role in the osseointegration process [128]. A number of studies also show that PGE2 osteoclastic activity [129, 130] and is an important marker associated with peri-implantitis [131, 132]. However, osteocytes can also release PGE2 under fluid-flow shear stress and PGE2 actually plays a more important role in bone formation than bone absorption [133]. PGE2 seems to have the ability to promote osteoblast differentiation [134] and induces bone formation [135] through the activates cyclic adenosine monophosphate (cAMP)/protein kinase A signaling pathway (cAMP/PKA) [133] and β-catenin signalling pathways [136]. In vitro studies also show increased production of PGE2 following osteoblast differentiation on rough surface implants [137]. IGF-1 is a positive regulator of bone formation and osteoblast activity [124, 125], and has been reported to be strongly expressed by osteocytes in response to under mechanical stimuli [138], and using human recombinant IGF-1 subcutaneous infusion can significantly inhibits alveolar bone loss following tooth extraction in rats [139]. The combination of IGF-1 with either platelet-derived growth factor (PDGF) or transforming growth factor beta 1 (TGF-β1) around a titanium dental implant significantly increases de novo bone formation within the peri-implant gap [140, 141]. OPN a member of the family of Small Integrin-Binding Ligand, N-linked Glycoproteins (SIBLING) [142] is a non-collagenous bone proteins that is secreted by both osteoblast and osteocytes and recruits MSCs to the fracture site and promoting bone formation [35, 143]. An in vitro study has shown that OPN is found at considerable levels on the rough surface implant in the early healing stages [123]. Bone morphogenetic proteins (BMPs) are another important group of growth factors enhancing bone formation and osteocytes secrete BMPs such as BMP-2 [126], BMP-3 [127], BMP7 [126] in response to mechanical stimuli. BMPs play an important role in osseointegration process [144-146]. And it was recently shown that BMP-7 was positive correlation with SLActive surfaced implant rather than the BMP-2, which significantly decreased in SLA surfaced implants [147]. Although there is no directly evidence of direct
osteocyte involvement with the osseointegration process, Gene array analysis in the early osseointegration periods shows the upregulation of soteocyte-related genes such as PHEX, DMP-1 DDk1 [148] indicating a complicated molecules crosstalk which is possibly controlled by osteocytes.

3.2.7 The important role of osteocytes in regulating osteoclast activity in osseointegration

Following implant placement, the process of modelling/remodelling of the surrounding bone continues throughout the life of the implant, with osteoblasts and osteoclasts working in a coordinated manner. Osteoclast activity can be observed around implant surfaces as early as 7 days following implant placement [100, 149]. Osteocyte apoptosis can occur at the site of micro-damage due to the implant placement and this is likely to recruit osteoclast precursor cells to the site and induce their differentiation [5, 150], thus initiating the bone resorption process. The receptor activator of nuclear factor kappa-B ligand (RANKL) is a key factor for osteoclast differentiation and activation [151]. Implant surfaces can affect RANKL-dependent differentiation in the osteoclast precursor cell line, RAW264.7 [101]. RANKL, along with the expression of the decoy receptor, osteoprotegerin (OPG), competitively binds to its receptor RANK and induces the differentiation/formation of osteoclasts [152]. The ratio of RANKL/OPG expression is responsible for regulation of osteoclast development and activity [152] and a recently study found the RANKL, RANK and OPG expression is activated immediately following the placement of implants [153]. Osteocytes are the chief source of RANKL in bone remodelling thereby controlling the formation of osteoclasts; although other cells types within bone may also contribute to the pool of RANKL [112, 151, 154]. In vitro results have shown that MLO-Y4 osteocyte-like cells can support the activation of osteoclasts and express RANKL and OPG [155]. Osteocytes undergoing programmed cells death also express RANKL by the release of apoptotic bodies, which in turn recruit osteoclasts [79]. Interestingly, stimulation of osteocytes by mechanical loading has been shown to increase matrix extracellular phosphoglycoprotein (MEPE), an associated motif peptide that is seen to possibly upregulate the expression of osteoprotegerin (OPG),
resulting in decreased osteoclastogenesis [156]. Using osteocyte-specific β-catenin-deficient mice (Ctnnb1(loxP/loxP); Dmp1-Cre) Kramer et al. found significantly decreased OPG expression coupled with increased RANKI/OPG ratio and enhanced osteoclastic activity. This suggests a dual role for osteocytes in the regulation of bone resorption. The mice had a dramatic reduction in both cancellous and cortical bone volume, whereas there was no effect on osteoblast or osteocyte numbers or any reduced cell viability [157]. Since the osseointegration process involves a RANKL-mediated mechanism and apoptotic osteocytes have the ability to simulate osteoclast function [98]. It can be inferred that insults such as trauma and heat from the implant placement process itself may initiate osteocyte changes that result in osteoclast activation.

### 3.2.8 The possible role of osteocyte specific-Wnt/β-catenin pathway in osseointegration

There are two Wnt-related signal pathways: the canonical Wnt/β-catenin pathway (β-catenin dependent) and the non-canonical pathway (β-catenin independent). The canonical Wnt/β-Catenin signalling pathway is the most extensively studied pathway and controls different aspects of bone metabolism by controlling differentiation of bone-forming osteoblasts and bone-resorbing osteoclasts [158, 159]. It is therefore not surprising that this is an important pathway in osseointegration. There are as many as 19 secreted Wnt signalling molecules, although it is still not clear exactly which of these are involved during bone formation [160]. Once an appropriate Wnt protein binds to its cognate receptor(s), which include low-density lipoprotein receptor-related protein (Lrp) 5 or Lrp6 and the receptors frizzled (FZD), it will trigger the downstream β-catenin pathway The Wnt/β-catenin pathway is one of the key pathways whereby osteocytes transmit mechanical signals into the terminal target cells [161]. Recently it was found that mechanical loading can affect the bone modelling and remodelling around implant [162] and some Wnt ligands, such as Wnt 10b, is upregulation during during osseointegration [163], and Wnt3a protein expression is increased by the micropitted/nanotubular surface topographies (MNTs) of titanium implants compared with smooth surface implant [164] and this protein has been shown to accelerate the rate of implant osseointegration [165]. There is also evidence to show that the surface topography
and chemistry of implants can affect Wnt signalling, and that implant surface properties can change the response of bone cells to Wnt factors [166]. Hydroxyapatite (HA) coated implants were placed in rat tibiae for 7 or 28 days and showed a significant increase of the Wnt co-receptors LRP5/6 and WISP1, a downstream target gene of canonical Wnt signalling, compared with uncoated Ti [167]. Hence, osteocyte-specific Wnt/β-catenin pathway is very likely to have an important role in osseointegration.

It is quite possible following mechanical loading that an osteocyte-specific Wnt/β-catenin pathway is activated by crosstalk with the prostaglandin pathway [89, 168], and that this leads to decreased expression of negative regulators of the pathway such as sclerostin (SOST) and dickkopf 1 (Dkk1) [161]. Research into negative regulators of Wnt/β-catenin pathway have focussed on SOST, Dkk1 and secreted frizzled-related protein 1 (SFRP1), which when expressed by osteocytes down regulate bone formation. Sclerostin, the product of the SOST gene, is a glycoprotein expressed and synthesized by osteocytes. Sclerostin is a competitive co-receptor of low-density lipoprotein receptor-related protein (Lrp) 5/6 (a positive regulator of Wnt/β-catenin pathway) and also acts as a BMPs antagonist; and therefore has the ability to inhibit osteoblasts activity [169]. Mechanical loading reduces the expression of sclerostin [170] and the use of sclerostin antibody treatment has been shown to accelerate and enhance the mechanical fixation of medullary titanium/steel or Polymethyl methacrylate (PMMA) implants in a rat model by increasing both cortical and trabecular bone volume [171, 172]. Sclerostin can also prevent the particle-induced implant loosening by promoting the bone formation at the same time inhibiting the bone resorption [173].

Dkk1 was originally identified as an antagonist of canonical Wnt signalling by acting as an Lrp5/6 inhibitor thereby inhibiting the differentiation of osteoblasts and bone formation [157, 174] and is also involved in mechano-transduction [175]. In vitro studies show that exogenous Dkk1 can cancel out the the differentiation enhancing effects on human MG63 osteoblasts of micropitted/nanotubular surface topographies (MNTs) titanium implants by reducing the activity of the canonical Wnt/β-catenin pathway [164]. Using a Dkk1 neutralizing antibody (Dkk1-ab) treatment in conjunction with steel implants screwed into rat
tibias, there was a significant increasing of bone formation and a significantly greater pull-out force was needed to dislodge the implant [175]. Sfrp-1 is another competitive antagonist of Wnt ligand binding, and in vivo loss of sfrp1 gene expression increases bone volume and density [176]. Sfrp1 is expressed mainly in immature osteocytes, and decreases in mature osteocytes Sfrp1 is expressed mainly in immature osteocytes, and decreases in mature osteocytes [177]. In vitro results show that loss of Sfrp1 promotes osteoblasts proliferation and differentiation into osteocytes [178]. Sfrp1/2 is also expressed at lower levels in MG63 osteoblasts cultured on MNTs titanium implant, culture conditions which lead to higher expression of Wnt3a [164].

Fig. 4 The theoretical relationship of implant with bone cells. The schematic shows how osteocytes are thought to affect osseointegration. Osteocytes control bone resorption by expressing RANKL and M-CSF, whereas the expression of OPG inhibits osteoclast activity. Osteocytes also secrete factors such as PGE2, NO, and ATP that can activate the Wnt/β-catenin signalling pathway and promotes bone formation by osteoblasts. On the other hand, osteocytes also release factors such as sclerostin, DKKs, and SFRPs, which have an inhibitory effect on Wnt/β-catenin signaling and results in decreased osteoblast activity.

3.2.9 A proposed mechanism of osteocytes in regulation of osseointegration [Fig. 4]

In the conventional understanding of the biology of implant placement and healing, the primary stability is very important for the success of bone healing around the implant. In
order to achieve primary stability, the implant bed needs to have a slightly smaller diameter than the implant, to achieve a fit between the implant surface and the surrounding bone. This will often produce mechanical stress to the surrounding bone tissue including nearby osteocytes, thereby triggering the mechanical stimuli and activate Wnt cell signalling pathway these osteocytes. The lacuno-canaliculur system of osteocytes is an ideal network for osteocytes to send signals out for MSC recruitment and osteoblast differentiation. Presumably, loading of the bone surrounding the implant when primary stability is acheived can affect the bone fluids that would otherwise flow freely through the existing lacuno-canaliculur system. Osteocytes are capable of sensing such changes through the cell body or its attached structures [114], and rapidly respond to the stress, including the opening of connexion 43 hemichannels, enhanced gap junction functions [74] and activation of the cell signalling pathways such as the Wnt/β-catenin, cAMP/PKA [161] and RANKL\OPG related pathways [151, 179]. A series of osteoblast related factors such as nitric oxide (NO), adenosine triphosphate (ATP), prostaglandin (PGE₂), IGF-1 and BMPs as well as osteoclast related factors such as RANKL and OPG will be released from the osteocytes to regulate osseointegration process.

3.2.10 Conclusion

Osteocytes play a dual role in both bone formation and bone resorption. The implant-healing process is similar in many ways to bone healing, including the activity of osteoblasts and osteoclasts. Exploring the mechanism of how osteocytes regulate the bone healing process around implants would significantly enhance our understanding of osseointegration of dental implants.
3.2.11 References


Chapter 4: Rat osteoporotic maxilla model study
Estrogen deficiency associated bone loss in the maxilla: a novel methodology to quantify the changes in the maxillary intra-radicular alveolar bone in an ovariectomized rat osteoporosis model

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(Tissue engineering accepted manuscript)


Suggested Statement of Contribution of Co-Authors for Thesis by Publication

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Prof. Yin Xiao

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4.1 Abstract

The effects of estrogen deficiency on bone characteristics are site-dependent, with the most commonly studied sites being appendicular long bones (proximal femur and tibia) and axial bones (vertebra). The effect on the maxillary and mandibular bones is still inconsistent and requires further investigation. This study was designed to evaluate bone quality in the posterior maxilla of ovariectomized rats in order to validate this site as an appropriate model to study the effect of osteoporotic changes.

**Methods:** Forty-eight 3-month-old female Sprague-Dawley rats were randomly divided into two groups: an ovariectomized group (OVX, n=24) and Sham-operated group (SHAM, n=24). Six rats were randomly sacrificed from both groups at time points 8, 12, 16 and 20 weeks. The samples from tibia and maxilla were collected for Micro CT and histological analysis. For the maxilla, the volume of interest (VOI) area focused on the furcation areas of the first and second molar. Trabecular bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and connectivity density (Conn.Dens) were analysed after Micro CT scanning.

**Results:** At 8 weeks the indices BV/TV, Tb.Sp, Tb.N and Conn.Dens showed significant differences (P<0.05) between the OVX and SHAM groups in the tibia. Compared with the tibia, the maxilla developed osteoporosis at a later stage, with significant changes in maxillary bone density only occurring after 12 weeks. Compared with the SHAM group, both the first and second molars of the OVX group showed significantly decreased BV/TV values from 12 weeks, and these changes were sustained through 16 and 20 weeks. For Tb.Sp, there were significant increases in bone values for the OVX group compared with the SHAM group at 12, 16 and 20 weeks. Histological changes were highly consistent with Micro CT results.

**Conclusion:** This study established a method to quantify the changes of intra-radicular alveolar bone in the posterior maxilla in an accepted rat osteoporosis model. The degree of the osteoporotic changes to trabecular bone architecture is site-dependent and at least 3 months are required for the osteoporotic effects to be apparent in the posterior maxilla following rat OVX.
4.2 Background

Osteoporosis (OP) is a systemic disease that affects over 200 million people worldwide [1, 2]. It is characterized by a decrease in bone mass and strength that predisposes the bone to fracture, and negatively influences the bone healing process [3, 4]. In order to better understand the biological mechanism associated with osteoporosis, ovariectomized rats have been widely used as a model to simulate human postmenopausal osteoporosis. Ovariectomized rat and osteoporotic human bone share many characteristics, including an increased rate of bone turnover with resorption exceeding formation, an initial rapid phase of bone loss followed by a much slower phase; greater loss of cancellous bone than cortical bone; and similar skeletal response to therapy with pharmaceuticals [5]. Other important reasons for utilisation of a rat model include cost-effectiveness, as well as ease of handling and housing, when compared with other models of osteoporosis, especially those involving large animals[6].

The healing of fractures of long and axial bones has been shown to be negatively affected by osteoporosis [4]. In relation to oro-dental conditions, clinical studies have revealed positive correlations between periodontitis and estrogen deficiency [7-10], tooth loss and osteoporosis [8, 11, 12], delayed alveolar bone wound healing and increased severity of residual alveolar ridge resorption caused by estrogen deficiency [13, 14].

The effects of estrogen deficiency on bone parameters such as size, mass and density are site-dependent [15]. The most widely investigated sites for osteoporotic bone loss are appendicular long bones (proximal femur and tibia) and axial bones (vertebra), and the changes reflected in bone morphometric parameters are well characterized in these areas [15, 16]. It appears that there is an inherent genetic difference between bones of cranial and non-cranial origin [17], which is not surprising given the differences in embryological origin and mechanisms of ossification. According to Ishihara et al, bone loss following ovariectomy predominantly occurs in those areas formed by endochondral ossification, such as the distal femur, rather than in areas formed by intramembranous ossification, such as the maxilla [18]. Mavropoulos et al. also reported a reduced response in the mandibular to estrogen deficiency
in rats, when compared to other skeletal sites [19], and other animal studies have produced similar results [20, 21]. However, some animal studies have reported positive correlation of osteoporosis and alveolar bone changes, as well as periodontal bone loss [7, 9, 10, 14]. Therefore, there is a clear need to clarify the effect of estrogen deficiency on maxillary and mandibular alveolar bones. Given trabecular bone is believed to be more susceptible to be affected by estrogen deficiency than cortical bone [22], maxillary bone, especially the porous, posterior maxillary region may be regarded as a more sensitive location for estrogen deficiency, compared with mandibular bone [23]. However, most researchers have used the rat mandible rather than the maxilla as the site of choice to study estrogen related bone loss following ovariectomy [19, 20, 24-26]. This may be due to the simpler geometry of the mandible, which makes it easier to determine bone volume when compared with the maxilla, which is characterized by irregular, more complicated roots and the presence of the maxillary sinus.

Traditionally, the main methods used for analysing bone characteristics are histomorphometry and/or 2 dimensional (2D) radiography (including Dual Energy X-Ray Absorptiometry, DEXA). DEXA mainly focuses on bone density (BMD) and is not an optimal tool when used in the presence of excess adipose tissue [27] and is also a challenge with growing animals [6]. On the other hand, histological assessment has the inherent limitation of only reflecting an isolated site, rather than whole bone changes [6]. 3D micro CT analysis has been used to overcome these limitations to obtain conclusive research outcomes [1, 28-30]. Although 3D Micro CT has been used by a few researchers to analyse mandibular bone [31, 32], it has not been used for analysis of maxillary bone changes due to the lack of method to extract the volume of interest (VOI) areas in maxilla. Therefore, this study was undertaken to compare maxillary and tibial bone morphometric parameter changes resulting from estrogen deficiency induced by ovariectomy. Micro CT and histological analysis were used to evaluate bone changes at different time points over a period from 8 to 20 weeks post-ovariectomy.
4.3 Materials and Methods

Animals

Forty-eight 3-month-old female Sprague-Dawley rats, weighing 232–267g, were obtained from an animal resource centre (SLAC Laboratory Animal Co. Ltd, Shanghai, China). The animals were randomly divided into two groups: an ovariectomized group (OVX, n=24) and Sham-operated group (SHAM, n=24). The ovariectomy operation was performed according to methods described in our previous study [33]. Briefly, the ovaries of the rats were exteriorized safely after ligature was placed at the end of fallopian tube; for the SHAM group, the same size of fats tissue near the ovaries was removed. The muscle and skin were sutured in layers. Six rats per group at different time point were sacrificed at 8, 12, 16 and 20 weeks, after ovariectomy or sham operation. One tibia and one maxilla from each animal were randomly selected for Micro CT analyses, while the contralateral bones were prepared for histological analysis. All animal research protocols were approved by the Animal Care and Use Committee of Fujian Medical University and are similar to our previous studies [33]. (Fig.1)

![Fig. 1: The research timeline. Animals were subjected to ovariectomy or sham surgery at the age of 12 weeks and sacrificed at 8, 12, 16 or 20 weeks after surgery.](image)

Micro CT evaluation

Bone histomorphometric parameters and the microarchitectural properties of maxilla and tibia were evaluated using a micro-CT system ((μCT 40; Scanco Medical AG, Bassersdorf, Switzerland)). The bones were scanned at an energy of 70 kV and intensity of 114 μA, with 300 ms integration time, resulting in 16-μm isotropic voxel size. For the scanned tibia, the volume of interest for evaluation of bone morphometric parameters was chosen over 200 slices, starting 25 slices distal from the growth plate. The trabecular bone compartment was
determined using a semi-automatic algorithm.

For the posterior maxilla, the scanned area of interest included three molars. The volume of interest (VOI) region focused on the furcation areas of the first molar and second molars. Initially, a circle with 40 pixel (0.64 mm) diameter was selected at the most coronal aspect of the root furcation, and after a further 40 slices apically a circle was selected with 60 pixel (0.96 mm) diameter. A third circle with the same diameter was then selected after another 60 slices and eventually, the VOI was defined by morphing across the slices between these three circles, forming an irregular, conical cylinder. Trabecular bone volume fraction (BV/TV, %), trabecular thicknesses (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and connectivity density (Conn.Dens) were determined for VOI using the micro CT evaluation software SCANCO (Scanco Medical AG, Bassersdorf, Switzerland) provided with the micro-CT system [34]. (Fig.2)

Figure 2: (a) shows the definition of VOI from microCT scans; (b) shows first (1st) circle with 40 pixel diameter from the beginning of furcation area underneath the molar (first circle); (c) shows second circle (2nd circle) with 60 pixel diameter after 40 sections from the first circle in furcation area; (d) is the trabecular bone fraction underneath this molar after the slices between these circles were morphed, resulting in an irregular, conical cylinder as VOI.
Histology

Samples for histological evaluation were fixed after sacrifice in 70% alcohol. Further processing of the samples was carried out according to routine methods. Briefly, the samples were dehydrated in a series of graded alcohol, then embedded in methylmethacrylate (MMA) (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. Slices of 10 to 15µm thickness were obtained using an Exakt cutting-grinding system (EXAKT Apparatebau, Norderstedt, Germany). Sections were stained using toluidine blue or Methylene Blue – Alizarin Red and scanned using Aperio microscope (Aperio, Vista, CA, USA). Trabecular bone volume fraction (BV/TV, %) at 12 weeks was analysed by the system’s image analysis software (Aperio, Vista, CA, USA).

Statistical analysis

Data are expressed as means ± standard deviations. One-Way ANOVA was used to compare body weights and identify significant differences between tibia and maxilla at different time points in the OVX and SHAM groups. p values of <0.05 were considered significant.

4.4 Results

Body Weight

Generally, ovariectomy resulted in weight gain. At baseline, there were no differences in weight between the two experimental groups. However, after ovariectomy, the body weights changed significantly. The weight of the animals from the OVX group was higher compared with the SHAM group at all evaluated time points (8, 12, 16 and 20 weeks respectively) (Fig.3).
Figure 3: Analysis of body weights showed significantly higher body weights in OVX treated rats from 8 weeks throughout entire experimentation period.

Micro CT Assessment

For the tibia, OVX rats had reduced quality trabecular bone structures compared with SHAM operated rats at both 8 and 12 weeks (Fig.4 a, b). After 8 weeks, BV/TV (Fig.5 a) was significantly decreased compared with the SHAM group. The same trends were seen for Tb.N (Fig.5 d) and Conn.Dens (Fig.5 e), as trabecular bone of the OVX group was significantly declined, compared with that of the SHAM group. For Tb.Sp (Fig.5 b), the OVX group achieved significantly higher values than the SHAM group, while no differences were found between the two groups for Tb.Th (Fig.5 c).

Figure 4: 3D micro CT (a, b) and histology images (c, d) of tibia at 8 weeks. Compared with SHAM group (b, d), OVX treated rats (a, c) had significantly less trabecular bone. Rats’ trabecular bone of tibia has significantly decreased (Scale bar=1mm).
Figure 5: Micro CT results of tibia showed significant differences from 8 weeks for the parameters BV/TV (a), Tb.Sp. (b), Tb.N.(d), and Conn.dens (e), but no differences could be determined for the parameter Tb.Th. (c)

In the maxilla, significant changes in bone density only occurred after 12 weeks, in contrast to the tibia where these changes were already evident at 8 weeks. The 3D images of the first (Fig.6a, b) and second (Fig.6 c, d) molar furcation areas at 12 weeks showed more porous structure in the OVX group (Fig.6 a, c) compared with the SHAM group (Fig.6 b, d). Compared with the SHAM group, both the first and second molars of the OVX group showed significantly decreased BV/TV (Fig.7a, 8a) from 12 weeks, and these changes were sustained through 16 and 20 weeks. For Tb.Sp (Fig.7b, 8b), the ovarietomy caused significant increases in bone morphometric values for the OVX group compared with the SHAM group at 12, 16 and 20 weeks. For the other parameters (Tb.Th, Tb.N, Conn.dens), the changes were not always consistent between the two molars at the same time points. For Tb.Th, at the first molar, the ovariectiony caused statistically significant reductions of trabecular numbers at 12, 16 and 20 weeks (Fig.7c), but for the second molar, significant changes were only observed at 16 and 20 weeks (Fig.8c). For Tb.N, there were significant differences at 12 and 16 weeks in the first molar (Fig.7d), but only at 12 and 20 weeks for the second molar (Fig.8d). For Conn.dens, the findings were more variable. At the first molar site, the OVX group had a higher value compared with the SHAM group at weeks 8 and 16 weeks (Fig.7e). These inconsistent changes were also measured at the second molar site, where OVX group had a
statistically significant increase in Conn.dens at week 16 and 20 (Fig.8e). The detailed evaluation of micro CT results was in supplemental data Table 1-3 (see appendix).

Figure 6: 3D micro CT images of the first molar (a, b) and second molar (c, d). Compared with SHAM group (b, d), the bone in OVX group (a, c) is more porous at 12 weeks.

Figure 7: Micro CT results of first molar showed significant differences from 12 weeks for the parameters BV/TV (a), Tb.Sp (b), Tb.Th (c) and Tb.N (d). However, for the parameter Conn.Dens (e), the differences between the two experimental groups were not consistent across the examined time points. Note the difference between the results for Tb.Th (c) between the maxilla here and the tibia (Fig.4c).
Figure 8: Micro CT results of second molar showed significant differences between the groups for the parameters BV/TV, Tb.Sp, and Tb.N. from 12 weeks after surgery. For Tn.Th, the difference between the groups was statistically significant from 16 weeks. Also for the parameter Conn.Dens, the differences between the two experimental groups were not consistent across the examined time points. Note the difference of index Tb.Th compared with tibia (Fig.4c).

Histological Assessment

The histology images of tibia at 8 weeks showed significantly more sparse trabecular bone in OVX rats compared with the sham group (Fig.4 c,d), which is consistent with the 3D micro CT results (Fig.4 a, b). In summary, these results provide evidence that bone loss due to estrogen deficiency induced by ovariectomy surgery affected long bone as early as 8 weeks.

The evaluation of the histology images from the maxilla showed that the trabecular bone was affected by the ovariectomy operation at week 12, with the OVX group having increased bone marrow volume (Fig.9a) compared with the SHAM group (Fig.9b), which was consistent with the micro CT’s longitudinal sections (Fig.9c=OVX, 9d=SHAM) and the 3D micro CT results (Fig.6). The analysis of trabecular bone area showed higher values for the SHAM group compared to the OVX group (Fig.9e, P<0.05). The microscopic evaluation of histological sections highly supported the findings from micro CT.
Figure 9: Histology images (a, b) and micro CT longitudinal sections (c, d) of Maxilla at 12 weeks showed similar changes. Compared with SHAM group, OVX group has been significantly lower bone volume (P<0.05), with less trabecular bone and more bone marrow (e). (Scale bar=2mm)

4.5 Discussion

The impacts of osteoporosis on the dental health of patients are not clear-cut. However, the negative impacts on the dental health of patients undergoing certain treatments for osteoporosis are known. The ovariectomised rat is a frequently used model for osteoporosis recommended by the U.S. Food and Drug Adminstration (FDA) [35] owing to advantages including similar changes in bone metabolism during estrogen deficiency compared with humans [5], easy and safe handling and a low cost of acquisition [6]. However, the question of whether the rat’s maxillary and/or mandibular bone can be used to study osteoporotic bone loss compared with the routinely used long bones or vertebrae is still a controversial issue [36]. In the present study, we examined the effects of ovariectomy induced estrogen deficiency on the bone of the posterior maxilla, with the aim of validating its suitability as a model for studying the effects of osteoporosis on oro-dental conditions and procedures.

Methods used for the analysis of bone density can be divided into destructive and non-destructive methods. Compared with the well-established method of histomorphometry, the newer non-destructive micro CT method is based on building full three-dimensional
structures of the sample [37]. The use of this method effectively avoids potential variability by observers [38] and accounts for bone parameters varying within a given specimen [39]. Dual energy X-ray absorptiometry (DEXA) is another method that is regularly used to investigate bone density. But due to the low resolution and the limitation of 2 dimensional imaging, DEXA is not able to determine microstructural parameters [21]. Theoretically, the ideal way to analyse maxillary bone using micro CT would be to include the whole bone and exclude the tooth roots. However, this is very difficult to implement because of the irregular shape of maxilla and tooth roots, as well as the presence of the sinus and nose cavities. These difficulties may explain the lack of studies evaluating maxillary bone changes resulting from osteoporosis in the literature. The furcation area, which is entirely made up of trabecular bone, is an important site to reflect the impact of osteoporosis on bone quality. The furcation areas are also involved in periodontal disease and act as a recipient site in dental implant placement. Therefore, the furcation areas of the first molar and second molars were selected for micro CT analysis, assuming that any changes to the trabecular bone in the area would be representative of changes in the remainder of the maxilla. The third molar was not included because of the large variation of roots between animals and the difficulties to generate consistent and reproducible evaluation.

From our results, both animal weights and the trabecular bone content of the tibia in the OVX group were significantly different at the first observation time point of 8 weeks. Ovariectomy caused accelerated food intake which resulted in body weight increase, which is consistent with other reports in the literatures [21]. Ovariectomy also caused significant tibial trabecular bone loss in the OVX group compared with the SHAM group from 8 weeks. Bone volume fraction (BV/TV), Tb.N, and Conn.Dens were significantly decreased compared with the SHAM group. This meant that at 8 weeks, induction of the osteoporosis model in the tibia could be deemed as being successfully achieved, as has been described previously [40].

However, in maxilla, the decrease in bone density did not emerge until 12 weeks, as opposed to 8 weeks in the tibia. From 12 weeks in the maxillary groups, BV/TV showed a
significant decrease and Tb.Sp increased significantly in the OVX group compared with the SHAM group. The furcation areas of the first molar and second molar in the OVX group showed more porous compared with the SHAM group. Histological analysis and micro CT results also showed the same trend. The apparent difference of osteoporotic effect on maxilla and tibia following ovariectomy can be explained by the different embryological origins and different ossification processes between the two anatomic locations [19]. Additionally, the asynchronism may be explained by inherent genetic differences between bone of cranial origin and bone of non-cranial origin [17]. Our research clearly demonstrated that an osteoporotic state was reached in the maxilla, albeit at a delayed rate concurrently, in the long bone.

Another interesting finding is that the parameters used for the analysis of bone tissue architecture did not reveal the same changes between tibia and maxilla. In the tibia, the trabecular bone thickness (Tb.Th) showed no obvious differences between the two groups. However, in the maxilla, OVX caused bone trabeculae to become significantly thinner than in the SHAM group and the differences did not emerge at the same time point in the two regions evaluated in the maxilla (between first and second molar furcation areas). Connectivity density (Conn.Dens) in the tibia of OVX animal decreased from 8 weeks, but in the maxilla, there was no regular pattern for either the first or second molar. We speculate that this could be due to the different endochondral and membranous bone formation between long bones and maxillar/mandibular bones [19]. The complexity of the masticatory force distribution between the different teeth could also result in subtle structural differences between the first and second molars.

The maxilla is an irregularly shaped bone due to the presence of the sinus and nasal cavity in the molar area, which makes the micro CT analysis challenging, because it is almost impossible to acquire a standard reproducible shape of VOI for all specimens to be analysed. Before we chose the furcation areas for analysis, we tried to assess the entire maxilla, except for the teeth. But we found that either tissue from the VOI was incorrectly omitted, or it was otherwise very challenging to obtain a consistent shape between different rats. Consequently
we selected the furcation area for the analysis. Initially we wanted to include the molar furcation areas from all three molars, but the roots of the third molar have significantly different shapes between animals, so we only selected the first molar and second molar for our analysis. By choosing a conical cylindrical shape for our VOI in the furcation area, defined by three circles with diameters of 40, 60, and 60 pixels respectively, we were able to completely exclude the roots. However, it should be noted that this method may result in the exclusion of some trabecular bone from the analysis. While this represents a limitation of our research, it is positively comparable with limitations faced by other researchers who have previously attempted to identify the changes of mandibular bone as a result of osteoporosis, whether it was based on 2D X-ray [41-43] or 3D CT [44-46]. All of these studies were challenged by the difficulty of acquiring a standard VOI for comparison between animals and between experimental groups. We believe that the creative method developed in this study for defining the VOI’s in the maxilla may also be used for other applications in bones of irregular shape.

4.6 Conclusion

It appears that the trabecular bone compartment in the tibia is more sensitive than the maxilla to changes in estrogen levels as induced in rats by ovariectomy, demonstrating that the effects of estrogen deficiency on trabecular bone architecture are site-dependent and at least 3 months are required for the osteoporotic effects to be apparent in the posterior maxilla following rat OVX. A key outcome of this study is the establishment of a method to reproducibly define a standardised VOI in the posterior maxilla for the evaluation of trabecular bone morphology, which may also be used for bones with irregular shape.
4.7 References


Chapter 5: Rat maxilla is a good model for dental implant research
Evaluation of the 1st maxillary molar post-extraction socket as a model for dental implant osseointegration research.

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*(Clinical Oral Implants Research accepted manuscript)*

**Suggested Statement of Contribution of Co-Authors for Thesis by Publication**

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5.1 Abstract

Objectives: Published information regarding the use of rat jawbones for dental implant osseointegration research is limited and often inconsistent. This study assessed the suitability and feasibility of placing dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model.

Materials and methods: Forty two customised titanium implants (2×3mm) were placed bilaterally in the maxillary first molar area of 21 Sprague-Dawley rats. Every animal received 2 implants. The animals were subsequently sacrificed at days 3, 7, 14, 28 and 56 post-surgery. Resin-embedded sections of the implant and surrounding maxilla were prepared for histological and histomorphometric analyses.

Results: The mesial root of the first molar in the rat maxilla was the optimal site to place the implant. Although the most apical 2-3 threads of the implant penetrated into the sinus cavity, 2mm of the remaining implant was embedded in the bone. New bone formation at day 7 around the implant increased further at day 14, as measured by the percentage of bone-to-implant contact (%BIC) and new bone area (%BA) in the implant thread chambers (55.1 ± 8.9% and 63.7 ± 7.7% respectively). There was a further significant increase between day 14 and 28 (p<0.05), however no significant differences were found between day 28 and 56 in either %BIC or %BA.

Conclusions: The mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research. Osseointegration following implant placement as measured by BIC plateaued after 28 days. The recommended implant dimensions are 1.5mm in diameter and 2mm in length.
5.2 Background

The widespread acceptance of dental implants as a feasible option for the rehabilitation of patients who have lost their teeth as a result of disease, pathology or trauma, has driven significant research activity aimed at improving their effectiveness. To this end, modification of the implant surface [1, 2], configuration [3] and/or implant material [4, 5], have all been rigorously assessed as a means to improve the ability of implants to osseointegrate. More recently, the undesirable effects of systemic disease (e.g. diabetes and osteoporosis) on the osseointegration of implants have also been examined [6-8].

In order to assess the effects of these parameters on osseointegration and bone regeneration, various animal models have been routinely used before proceeding to human clinical trials. Small animals such as rodents (mice, rats, rabbits) are often used before moving to larger animal (dogs, sheep, pigs, primates) studies. Of these animal models, rats are the most frequently used medical model compared with other animal models [9], not only because of their cost effectiveness, easier handling and lesser laboratory space requirements, but also because they can be used to mimic human diseases such as osteoporosis [10] and diabetes [11, 12]. Notwithstanding these advantages, there are still some significant limitations in the use of small animal model systems in dental implant osseointegration research, such as technical difficulties associated with anatomical constraints [13], and the identification of a suitable implant site [13-15].

Historically, the most common site for placement of a dental implant in small animal models such as the rat has been the femur or tibia [7, 16-18] as these sites are relatively easy to access surgically and have sufficient amounts of bone tissue to embed the implant. However, significant differences in the embryological origin and ossification processes of long bones in comparison to craniofacial development [19, 20] warrants caution in translating the results obtained in a long bone model to the oral environment.

While a number of previous studies have utilised the rat maxilla to assess the osseointegration of dental implants, differences in the site and implant used make it difficult
to assess the validity of this model [13-15, 21-35]. Of these studies, most of the implant sites selected were in the first molar area and the diameter of Implants used varied between 1 and 4.5mm. Methodology regarding implant placement however was often deficient and the results rarely mentioned the complications / limitations relating to the use of the maxillary site for implant placement. Moreover, only a few of these studies used resin embedded samples to analyse the degree of osseointegration as measured by the percentage of bone to implant contact (%BIC).

The aim of the present study therefore, was to comprehensively assess the temporal changes in bone-implant contact during implant osseointegration in the rat 1st maxillary molar post-extraction socket in order to establish its suitability as a model for dental implant research.

5.3 Materials & Methods

Animals

Twenty three three-month-old female Sprague-Dawley rats, weighing 245–279g were obtained from the Animal Resource Centre (Murdoch Drive, Murdoch, Western Australia). Two rats per cage were housed at the University animal facility in temperature (22±2°C) and humidity (50±20%) controlled rooms with a 12 hr light and dark cycle. Commercial laboratory rat chow and water were available ad libitum throughout the study. The animals were acclimatised within the facility for one week before any surgical procedures were performed. The Griffith University Animal Ethics Committee (DOH/01/4/AEC) approved the research protocol.

To identify potential implant sites, two of the rats were sacrificed by anaesthetic overdose and their maxilla scanned by micro CT (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland). The ultimate implant design was subsequently based on these micro-CT analyses.

Implants
Moderately rough (Ra ≈ 1.27μm) titanium screw implants were custom produced by Southern Implants Ltd (Irene, South Africa). The implants (2mm in diameter and 3mm long) were produced from Type IV commercially pure titanium and using the same processes as those utilized in commercially available implants.

Surgical procedures

The surgical procedures are shown in figure 1(a-d). Twenty-one rats were anesthetized by Isoflurane (1–3%) inhalation and kept on heating pads to maintain body temperature during surgery. The oral cavity was first cleansed with 0.12% chlorhexidine gluconate before using a retractor to open the mouth and a crestal incision (approximately 2mm long) made at the mesial aspect of the first maxillary molars. A full thickness mucoperiosteal flap was raised in order to identify the most mesial root of the tooth. The 1st molar teeth were then extracted using a modified spatula and toothed forceps that were used as a root elevator and extractor forceps respectively.

The mesial root sockets were then inspected and a small dental curette was used to remove any residual root fragments. Only the mesial root needed to be completely removed for implant placement.

Initially, a 1.2mm diameter pilot drill (Southern Implants, Ltd., Irene, South Africa) was used to create the osteotomy to a depth of 2.5 - 3.0mm with a rotary speed not exceeding 1500 rpm. The final osteotomy was completed with a 1.8 mm diameter drill (Southern Implants, Ltd., Irene, South Africa). All osteotomy procedures were performed with copious saline irrigation. An implant was placed into each socket with a torque of 15-20Ncm until the most coronal parts of the implant coincided with the bone crest and primary stability was achieved. The flap was then closed with resorbable sutures (5/0 Vicryl) and the implant was fully submerged. No cover-screw or a healing abutment was used in this procedure.

The rats were then allowed to recover on the thermal pad. Post-operative analgesia (buprenorphine 0.01 - 0.05mg/kg and carprofen 4 - 5mg/kg) and antibiotic cover (enrofloxacin 2.5mg/Kg) were administrated by intraperitoneal injection immediately after the surgery and continued daily for three days post-operatively. After surgery, the rats were
given standard rat chow and water ad libitum. The animal’s weight was monitored daily to determine if there were any complications in feeding as a result of the bilateral molar extraction and implant placement.

Fig. 1. Shows the surgical procedures. (a) and (b) extraction of the teeth. (c) preparation of the osteotomy. (d) implant placement at level of the alveolar bone. (e) and (f) x-ray of implant in position.

Histological Preparation

Animals were sacrificed after 3, 7, 14, 28 and 56 days of healing (n=3 for days 3 and 7, n=5 for days 14, 28 and 56). Specimen blocks of the maxilla surrounding the implants were carefully harvested using a diamond-tipped circular saw with irrigation and immediately fixed with 4% paraformaldehyde for 48h at 4°C. The samples were then dehydrated in a graded series of ethanol before embedding in methylmethacrylate resin (Technovit® 7200 VLC, Heraeus Kulzer, Dormagen, Germany).

Sections 140 - 200 microns thick of the maxilla containing the implant were cut buccopalatal and parallel to the long axis of implant using the EXAKT cutting system. The sections were prepared as close to the centre of the implant possible which allowed 2 sections per implant to be prepared. These sections were then gradually ground to 30-microns in thickness.
using the EXAKT grinding system (EXAKT Apparatebau, Norderstedt, Germany) [7, 36]. The sections were stained with methylene blue-alizarin red S and scanned using the Aperio ScanScope CS at x40 magnification (Aperio Technologies Inc., Vista, CA).

**Histomorphometric analysis**

The BIC and BA were traced from the scanned images. Histomorphometric analysis of each specimen was then determined using ImageScope™ software (Aperio Technologies Inc). The percentage BIC is defined as the ratio of the sum of the implant surface in direct contact with bone tissue and the total length of the implant adjacent to native bone (Fig. 2). The bone area (BA) was defined as the fraction of mineralized bone tissue excluding any non-mineralised tissue within the threaded areas of the implant that were adjacent to native bone. The dimensions of the native bone from both buccal and palatal aspects were also measured.

![Diagram of histomorphometric analysis](image)

**Fig. 2.** The percentage BIC is defined as the ratio of the sum of the implant surface in direct contact with bone tissue (total length of the red dashed lines) and the total length of the implant (total length of the green dashed lines) adjacent to native bone. The adjacent native bone is shown as the areas between A & C and B & D. Any bone formation in the sinus area (blue area) is excluded.

**Statistical analysis**

Data is expressed as the mean +/- the standard deviation. The data were tested for normality using the Kolmogorov-Smirnov test, while the homogeneity of variance was
verified using Bartlett’s test. Subsequently One-Way ANOVA was used to compare differences in the amount of BIC and BA at the sacrificial time-points using Tukey’s HSD test. A p-value of <0.05 was considered significant.

5.4 Results

Anatomy and Micro-CT Analysis

Analysis of the initial two rat skulls (Fig. 3a, b) showed that there were three aligned molars posterior to the base of the zygomatic bone. The alveolar bone was obviously protuberant on the buccal side in order to accommodate the anatomy of the roots. No boundary between the sinus and nose cavity was observed and they appeared to merge to form a single cavity. Two large dehiscences (bony slits) were located oblique and anterior to the first molar. The widest horizontal dimension was located anterior to the maxillary first molar where the bone was approximately 3mm wide. Micro-CT analysis (Fig. 3c) demonstrated that the first molar in the rat maxilla has five roots, the largest of which was the mesial root with a length of 3mm and a diameter of 1-2 mm (Fig. 3d).

Fig. 3. Shows the anatomical relationship of the first molar and its mesial root(yellow line) with the maxilla. (a) shows the outline of maxilla, (b) shows the molar area of maxilla, red dotted line shows the widest width of the alveolar bone is located between the bottom of zygomatic arch and mesial root of first molar. Purple arrow shows the dehiscence in the anterior palate. (c) Longitudinal micro CT image of
maxilla shows the mesial root of the first molar is the largest of the roots. (d) shows the length of mesial root is nearly 3mm.

Based on these morphometric measurements, 2mm diameter x 3mm long customised implants were manufactured by Southern Implants Ltd (Irene, South Africa) with the same material, surface modification and manufacturing processes used in full sized human implants (Fig. 4). Subsequent morphological analysis of the maxilla from rats that received implants however, demonstrated that in some animals, the vertical dimension of the alveolar bone in this region was approximately 2mm. This meant that the most apical 2-3 threads of the implant could penetrate into the sinus cavity (Fig. 5).

Fig. 4. Customized moderate rough (Ra=1.27μm) surface implant made according to the anatomical characteristics of the mesial root of the first maxillary molar. (a) shows the design draft of the implant, (b) shows the implant surface under scanning electron microscope (SEM).

Fig. 5(a). Morphometric analysis shows the implant placed into the first molar position. (b) shows the alveolar bone height near the implant position was nearly 2mm. (c) shows 2-3 threads of the implant
penetrated into the sinus. (d) shows the implant was placed slightly to the palatal side. The ideal position should be as indicated by the yellow circle (i.e. more towards the buccal than palatal side).

**Histological and Histomorphometric analysis**

Post-operatively, all animals tolerated the surgical procedures without any obvious complications. After 3 days of healing, histological analysis showed that the implant remained in close contact with the walls of the recipient bone but no obvious new bone formation around the implant was observed. Whilst some pieces of fractured bone tissue were observed within the thread areas of the implant, no bone resorption was apparent (Fig. 6a). After 7 days of healing, new bone formation, both directly on the implant surface and from the parent bone was observed. While pieces of the fractured old bone still remained in some of the inter-thread areas, within the new bone, osteoblasts could be clearly identified. Bone healing at this time however was not completely consistent as some areas of the old bone around the implant showed bone lacunae that could have been the result of osteoclastic activity (Fig. 6b).

![Fig. 6](image)

Fig. 6. Shows new bone formation at day 3 (a) and day 7 (b). At day 3, the implant was mechanically integrated with bone tissue (green arrow), with pieces of fracture bones seen in-between the threads. At day 7 new bone had grown into some threaded areas (blue arrow) (bar=200µm).
After 14 days of healing, new bone formation was observed both on the implant surface and at the interface of the resorbed old bone (Fig. 7a). There were abundant osteoblasts located in the new bone surface and many osteoclast lacunae were still clearly present, although fewer than observed at day 7 (Fig. 7b, c). The mean percentage bone-to-implant contact (%BIC) and bone area (%BA), in the threaded regions of the implant at this timepoint was 55.1 ± 8.9% and 63.69 ± 7.7% respectively.

Fig. 7(a). Shows the day 14 histology results. More new bone was seen compared to day 7. (b) There were obvious osteoblasts arranged in the new bone surface (red arrows). (c) In the old bone areas, osteoclasts could be clearly seen (green arrows). (a, bar=200µm; b&c, bar=50µm).

The mean BIC (71.1 ± 13.7%) continued to increase significantly (p<0.05) over the next 14 days. Similarly, the percentage of new bone area (77.1 ± 5.3%) within the threaded chambers of the implant at day 28 also increased significantly compared with day 14 (p<0.05) (Fig. 8). Compared with the appearance of the newly formed bone at day 14, the woven bone in the threaded areas also appeared more dense and mature at day 28 (Fig. 8a, b).

At the final healing time-point 56 days post-surgery, the bone around the implant could now be considered mature. Osteoblasts and osteoclast lacunae only appeared in areas undergoing remodelling. Some samples clearly showed the border between the old and new
bone, but generally the osteocyte arrangement was more regular compared with day 28 (Fig. 8c,d). Furthermore, there were no significant further changes in either %BIC or %BA at day 56 compared with day 28 (Fig. 8 e,f).

A notable observation was that, depending upon the individual animal and the precise positioning of the osteotomy, the implant could penetrate into the sinus floor leaving only 2mm of the remaining implant embedded in the bone (Fig. 4, 9a). Histological analysis of the perforated areas however showed that there was usually obvious new bone formation around the sinus-embedded apical region of the implant (Fig. 9a). The average height of the maxillary bone on the buccal (1.8 ± 0.3mm) and palatal (1.5 ± 0.2mm) sides of the implant were also shown to be significantly (p<0.05) different (Fig. 9b).

Fig. 8(a, b). Histological results day 28. (c, d) Histological results day 56. At day 56, the bone appears to be more dense and mature. The bone between the original alveolar bone and that between the implant threads (between the green and blue lines) can be seen to be becoming gradually more mature. The osteocyte arrangement also appears to be better than that at day 28. (e, f) Compared with day 14, the %BIC and %BA were significantly increased (*p<0.05), but there were no further significant differences between day 28 and day 56 (a & c, bar=200µm; b & d, bar=100µm).
Fig. 9(a). Shows ~2mm of the implant was embedded into the bone tissue. There was also new bone formation seen around apical area of the implant. (b) the height of the alveolar bone on the palatal side was significantly lower than the buccal side (*p<0.05). (Bar=1mm).

Fig. 10(a). Shows the implant position was located slightly in platal side. (b) shows the correct position and the improved result (c) (bar=1mm).

5.5 Discussion

The rat is a widely used animal in dental research [26-28, 37]. The jaw bones have been used to model many human orofacial clinical situations, such as periodontitis [38], orthodontic tooth movement [39], periodontal regeneration [40], and the relationship between systemic diseases (e.g. osteoporosis [41] and diabetes [38]) with periodontal disease. However, there is inadequate published literature describing the use of the rat maxillary bone in dental implant research. The recent consensus statement from the Eighth European workshop on preclinical in vivo research (ARRIVE guidelines) concluded rodents were primarily used for osseointegration research when examining the effects of systemic disease.
When healthy animals were used, the predominant implant sites were extra-oral, i.e. tibia and femur [42].

There are two pre-clinical studies that specifically recommend the use of the rat maxilla as acceptable implant placement sites for dental implant research. Kavanagh et al. (1985) used a specially designed implant placed into the extraction socket of the second maxillary molar. A splint was then used to keep the implant stable during the healing phase. In a later study, Karimbux et al. (1995) placed a 1 x 2mm implant into the maxilla without extracting the molars. Both of these studies raised the issue of the difficulties associated with the surgical procedures and subsequent histological preparation and analysis. However both recommended the use of the maxilla as a useful site for implant placement. The methodology proposed in these studies however is of limited value today given recent developments in both implant technology and histological analysis using resin-embedded sections. Another dilemma for researchers is the selection of the ideal implant position as these two studies used different implant placement positions within the maxillary alveolar bone. Kavanagh et al. (1985) recommended using the second maxillary molar site following extraction whilst Karimbux et al. (1995) used a site anterior to the first molar. Interestingly, a majority of subsequent pre-clinical studies that used the maxilla, chose to select the first molar site rather than either of the positions recommended by the above authors [14, 15, 21-27, 29-35]. Furthermore, most of those studies also omitted to report a detailed methodology and/or histological and histomorphometric analysis.

Long bones such as the femur have been the most commonly utilized site for dental implant research due to their relatively easier surgical access compared to the oral cavity. However there are several issues with the use of long bone as a research tool for the study of dental implant osseointegration. It is impossible to simulate the oral environment in the long bone model and hence it is difficult to extrapolate the results to clinical situations. There are also multiple common oral environmental factors that cannot be simulated in the long bone model such as masticatory function, interactions with oral bacteria and the effects of saliva, which may have important influences on osseous healing and the osseointegration process.
Perhaps more importantly, there are fundamental differences in the bone tissues themselves in relation to developmental origin and ossification mechanism. Embryologically, long bones arise from the mesoderm whereas the craniofacial bones arise from the neuroectoderm [43]. The ossification mechanism (intramembranous for maxilla compared to endochondral for long bones) and response to hormones are also different between these two types of bones and it is believed that craniofacial bones are less sensitive to hormonal changes than the extra-cranial skeleton [44]. For example, studies that have used animal long bones have showed that osteoporosis negatively influenced osseointegration. However similar studies when repeated in alveolar bone found that there was no effect on osseointegration [32, 45]. Furthermore, evidence has shown that there is a higher mineral density and calcium concentration in intramembranous bones compared with long bones in both humans and animals [46-48] along with bone matrix compositional differences [49]. It is also noteworthy that the morphology of osteocytes found in intramembranous bones is significantly different from that of long bones, being more round in shape [50]. These may reflect inherent genetic and/or epigenetic differences between bones of craniofacial and non-cranial origin [51]. On the whole, this evidence suggests that a craniofacial alternative to using long-bones in the rat as a model for dental implant osseointegration research is warranted.

The morphological and micro-CT analysis in the present study demonstrated that the anatomy of the rat maxilla is relatively complex. The mesial root of the first molar was determined to be the most suitable site for dental implant placement. However the two fenestrations in the palate located oblique to the front of the first molar result in significantly thinner bone on the palatal side compared to the buccal side. Hence, when preparing the implant socket, the drill position should be slightly more towards the buccal than the palatal side, as indicated by the yellow circle in figure 5d.

The decision to use a 3mm long implant in this study was based on the dimensions of the mesial root of the first molar obtained from the preliminary morphometric analyses (Fig 3.). While the post-surgical histology results often showed that the sinus was penetrated by the
apical aspect of the implant, the implant placement protocol necessitates the engagement of the cortical plate of the sinus floor in order to achieve primary stability. Thus, we suggest that the osteotomy position and angulation should be more towards the buccal bone, at least 0.5mm away from the mesial root extraction socket, rather than just following the direction of the extraction socket (Fig. 5d and Fig. 10). Also a shorter implant is recommended which would engage more with the buccal bone, decreasing the incidence of sinus perforation.

Fracturing of the tooth roots was a common observation. If the implant is placed in contact with the remaining roots, there is a significant possibility of forming an encapsulation from the PDL thus reducing the degree of osseointegration. However we found the distance between the distal roots and mesial root (on average ~ 2 mm) makes this an unlikely event (Fig. 3).

The percentage of bone to implant contact (%BIC) is routinely used to quantitate osseointegration. However, a variety of methods have been used to assess bone to implant contact [52-54] and the methodology used to measure this key parameter is variable [15, 24, 29]. While the implant used in this study is slightly longer than the available alveolar bone height, by only assessing implant contact with the adjacent native bone, this will exclude any bone formation associated with the sinus perforation (Fig. 2).

There are many factors to consider when selecting an appropriate animal model for dental implant research. Most previous animal studies have used larger animals such as the pig, dog, sheep and even primates [55-59]. The advantages for these models includes a similarity in bone composition and bone density [60], as well as similar bone remodeling and bone turnover compared with humans [61-65]. Beside these, another clear advantage of such animal models is the similar size of the jaw-bones compared with humans, thus permitting the use of commercially available implants. This infers that the results obtained are more likely to mimic those expected in humans.

However, larger animals are usually more expensive, require larger and more sophisticated animal facilities, and have a higher maintenance cost, which partly inhibits their availability for research. In comparison, smaller animals will always be more cost-effective,
easier to house and handle [10] and allow easy chronological observation and larger samples numbers [66]. In the case of rats, an additional advantage is the ability to readily simulate various systemic diseases that could influence bone quality and osseous healing processes such as osteoporosis [10, 42, 53] Importantly, it has been demonstrated that rats have very similar trabecular bone remodeling to that in humans [67].

The demonstration of similar results to those seen with larger animals would therefore indicate that the rat maxilla is indeed a good model for dental implant research. The histological results of this study show that the first molar maxillary site allows for customized implants to be embedded approximately 2mm into alveolar bone. By day 7 of healing, there was clearly new bone formation around the implant with significant osteoblast and osteoclast activity, which is in agreement with previous studies [31]. New bone formation continued to form until day 28 after which there were no further significant increases in either the percentage BIC or BA. Previous studies in a primate model, demonstrated a similar level of BIC at 67.3% in the maxilla following the establishment of osseointegration after nine months [58]. Considering the differences in metabolic rates [68], 28 days of healing in rats approximately correlates with the results obtained in primates after 1 year. This is additional evidence supporting the proposed maxillary site in the rat as an alternative model for dental implant research.

5.6 Conclusion

We found the mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research. 28 days of healing can be considered as an appropriate end point to assess the degree of osseointegration as measured by percentage bone-to-implant contact (%BIC) and bone area (%BA). The recommended implant dimensions for this particular site in the rat is 1.5mm in width and 2mm in length.
5.7 Reference


Chapter 6: The ultrastructure of osteocytes on osseointegration
The Ultrastructural Relationship between Osteocytes and Dental Implants

Following Osseointegration.

Zhibin Du, MDSc; Saso Ivanovski, MDSc, PhD; Stephen M. Hamlet, PhD; Jian Q. Feng, PhD; Yin Xiao, MDSc, PhD*

(Clin Implant Dent Relat Res accepted manuscript)


Suggested Statement of Contribution of Co-Authors for Thesis by Publication

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6.1 Abstract

Background: Osteocytes, the most abundant cells in bone, have multiple functions including acting as mechanosensors and regulating mineralization. It is clear that osteocytes influence bone remodeling by controlling the differentiation and activity of osteoblasts and osteoclasts. Determining the relationship(s) between titanium implants and osteocytes may therefore benefit our understanding of the process of osseointegration.

Purpose: The aim of this study was to visualize the ultrastructural relationship between osteocytes and the titanium implant surface following osseointegration in vivo.

Materials and Methods: Titanium implants were placed in the maxillary molar region of eight female Sprague Dawley rats, aged 3 months old. The animals were sacrificed 8 weeks after implantation and undecalcified tissue sections were prepared. Resin-cast samples were subsequently acid-etched with 37% phosphoric acid prior to examination using scanning electron microscopy (SEM).

Results: Compared with mature bone where the osteocytes were arranged in an ordered fashion, the osteocytes appeared less organized in the newly formed bone around the titanium implant. Further, a layer of mineralization with less organic components was observed on the implant surface. This study has shown for the first time that osteocytes and their dendrites were directly connected with the implant surface.

Conclusions: This study shows the direct anchorage of osteocytes via dendritic processes to a titanium implant surface in vivo. This suggests an important regulatory role for osteocytes and their lacunar–canalicular network in maintaining long term osseointegration.
6.2 Background

Osteocytes embedded in bone tissue are the longest-lived and most abundant cell type in adult bone, comprising 90~95% of all bone cells. [1] These cells arise from matrix-producing osteoblasts passively embedded in the osteoid/matrix produced by neighboring cells. [2] Recently, mounting evidence supports a prominent and multi-factorial role for osteocytes in bone homeostasis. They may act as a mechanosensor perceiving strain signals and developing micro-cracks through their cell body, dendritic processes or the bending of cilia. [3, 4] [5] They may also react to hormonal changes such as estrogen deficiency, which through an increase in osteocyte apoptosis, triggers bone remodeling and resorption. [6] Osteocytes may also play a role in mineral (phosphate) regulation and biomineralization via the action of osteocyte-specific proteins such as PHEX, DMP-1, MEPE, and FGF-23. [7] It has also been shown that osteocytes play a very important role in bone remodeling through the actions of the Wnt/β-catenin pathway regulators Dkk1 and sclerostin, both of which are highly expressed in osteocytes. [6] Previously thought of as a “passive placeholder in bone”, [8] it’s now being increasingly acknowledged that the osteocyte plays a key regulatory role in osteoclast and osteoblast activity and mineral metabolism.

The dental implant has become a popular and predictable technique for replacing missing teeth. This treatment relies on the process of osseointegration whereby the titanium is bound to bone tissue without any intervening soft tissue. [9] However, the biological mechanisms responsible have not been fully elucidated, especially with regard to the possible role of osteocytes in the maintenance of osseointegration. [10] Moreover, the relationship of osteoblasts/osteocytes with the implant surface and the ultrastructural details of the bone-implant interface are not fully understood. The ‘gold-standard’ for evaluating osseointegration is histological staining and observation using light microscopy (LM), [11] whereby non-
The ultrastructure of osteocytes on osseointegration

decalcified ground sections of resin embedded samples are used to measure the bone contact ratio (BCR) or bone-implant-contact (BIC). [12] Scanning electron microscopy (SEM) in the backscatter electron emission (BSE) mode can also be used for the measurement of BCR/BIC to examine the early implant-bone healing process in vivo. However, one of the limitations of the LM and BSE-SEM methods is the inability to identify different types of bone cells in vivo. Transmission electron microscopy (TEM) is a better way to visualize the cells around the implant, but this technique can only examine the interface without the implant being present, and only allows observation in two dimensions within a very small area. Therefore, a method which can visualize the ultrastructure of the implant-bone interface in three-dimensions (3D) would be beneficial in facilitating the study of the relationship between bone cells and the implant surface.

Resin-cast methods utilizing acid etching were originally used to investigate the 3D structure of osteocytes and their lacunar–canalicular network (LCN) at the outer border of fractured bone samples. [13-15]. Historically, fracture techniques combined with an acid etching protocol do not always adequately maintain the integrity of osteocytes and their LCN structure. More recently, Feng et al. used a 37% phosphoric acid etching treatment on the surface of bone tissue embedded in MMA (methyl methacrylate) to acquire a clear 3D SEM image of osteocytes with their LCN structure. [16] This approach provides a novel method to study the delicate and complex ultrastructure of osteocytes and their associated LCN within bone tissue. Since osseointegrated dental implants are in direct contact with bone without any intervening soft tissue, examination of osseointegrated implant samples using the acid-etched resin-cast implant can be used to better understand the implant-bone interface.

In vitro studies have shown that osteoblasts attach to implant surfaces where they secrete osteoid onto the surface.[17, 18] As osteocytes are the terminal cells of
osteoblasts, we hypothesise that osteocytes may remain in close contact with the implant surface during osseointegration, where they are then able to play an important regulatory role in the modeling and remodeling processes of osseointegration. The aim of this study therefore was to investigate the feasibility of using the resin-cast osteocyte method to observe the relationship between the LCN (including osteocytes) structure and the implant surface at the ultrastructural level in osseointegrated implant samples.

6.3 Materials and methods

Animals.

Eight 3-month-old female Sprague-Dawley rats, weighing approximately 265–300g, were obtained from the Shanghai animal resource center (SLAC Laboratory Animal Co. Ltd, Shanghai, China). The Animal Care and Use Committee of Fujian Medical University approved the animal research protocol. The protocols used are similar to previously published research. [19]

Implants and Implant placement

Screw shaped micro-rough surface modified implants (2 mm in diameter x 3 mm long, Ra=1.55μm) were obtained from Southern Dental Implants (Irene, South Africa). Implant placement was performed under general anesthesia. Briefly, one maxillary first molar was extracted from each animal followed by immediate insertion of the implant into the freshly extracted socket. The animals were sacrificed at 8 weeks using an overdose of ketamine.

Resin Embedding and Acid-Etching

The non-decalcified maxillae containing the implants were fixed in 4% neutral formalin for 48 hours. The specimens were dehydrated in a series of graded alcohols and were embedded using Technovit 7200 resin. Ground sections of the calcified samples were prepared as previously described. [19] Briefly, the specimens were sectioned using a diamond saw (Exakt-Trennschleifsystem 300CP, EXAKT®)
Apparatebau GmbH & Co KG, Norderstedt, Germany) at two points equidistant from the middle and perpendicular to the long axis of the implants. One of the sections was ground to a 30 µm thickness for histological assessment, while the other section was first ground using 1200 grit sandpaper and then polished with diamond suspensions (6, 1, and 0.25 µm particle size) and prepared for the resin-cast osteocyte technique as previously described. [16] Briefly, the surface of the polished sections was first acid etched with 37% phosphoric acid for 10 seconds then immersed in 5% sodium hypochlorite for 5 min and air-dried overnight. The sections were coated with gold and examined using a Carl Zeiss SIGMA VP field emission scanning microscope (FE-SEM; Carl Zeiss, SMT GmBH, Oberkochen, Germany) operated at a voltage of 15 or 20 kV. The focus of the ultrastructural analysis was the interface between bone and the implant surface. Elemental analysis was performed using the polished samples coated with gold with the same scanning microscope.

6.4 Results

Under light microscopy, histological images showed that most of the implant surface was covered with new bone lamella that was connected to the pre-existing bone by newly formed trabecular bone (Fig.1a) There was no observable gap between the implant and bone, even under high magnification. Multiple osteocytes could be seen in the new bone surrounding the implants although it was difficult to visualize the LCN structure (Fig.1b)
Figure 1. (a) Bone-implant interface. (b) At higher magnification, there is no visible gap between the bone tissue and implant surface. Dotted line shows the border of new bone and old bone. In the newly formed bone area, osteocytes with nuclei can be observed.

BSE-SEM observation under low magnification also showed the bone to be in close proximity to the implant surface, with no gap evident between the implant and bone tissue at the interface (Fig.2a). This is consistent with the light microscopy observations. However, under higher magnification a small gap of approximately 2-2.5 µm is evident in some areas at the interface between the implant and bone (Fig.2b). This is most likely a result of resin shrinkage during polymerization resulting in a fracture between the implant and the bone tissue.

Figure 2. Backscatter image of an osseointegration using SEM. (a) Under low magnification, the histological image shares many similarities to those of the light microscopy,
where bone is in close contact to the implant surface. (b) Under higher magnification, there is a 2-2.5 µm gap seen between the implant and bone interface in some areas. This gap as a result of processing however was inconsistent as in some areas there was no shrinkage observed.

Following acid-etching of the undecalcified specimens, under low SEM magnification it was apparent that most of the calcified component of the bone matrix had been removed leaving the osteocytes exposed. It was observed that the osteocytes were arranged in an orderly manner in the bone distant to the implant. This bone is likely to be mature tissue that was not involved in the healing process associated with implant placement and osseointegration. However, new osteocytes located adjacent to the implant appear to have a disorderly arrangement (Fig.3a&b). There are more osteocytes in the newly formed bone areas compared with the old trabecular bone, especially in the area surrounding the implant thread (Fig.3a&3c). The residual irregular, intact tissue apparent in the images is likely to be non-mineralized matrix, vessels or marrow cavities which were not affected by the acid-etching process (Fig.3a).

Figure 3. Low SEM magnification shows the outline of bone tissue. Note that osteocytes in old bone area appear well-ordered (a) red dotted line shows the border of old bone and new bone; yellow dotted line shows osteocytes with ordered arrangement) compared with osteocytes in new bone area (thread area). The irregular, intact tissue includes non-mineralized matrix, vessels (red box show vessels in the bone tissue) and bone marrow cavities (BM). (b) shows the unordered osteocytes between threads (arrows indicate osteocytes). Cells become disorganized in the newly formed bone, especially between the threads. (c) Compared with the old bone area, average osteocyte numbers (Avg OS.N) were significantly higher in the new bone areas (p<0.05).
Under higher magnification, the osteocytes and the associated LCN could be clearly visualized around the implant (Fig.4). Osteocytes were situated in the cavity of their lacunae, which is evident as a relatively dark area after the acid-etching process. Osteocytes were connected with each other via a dendritic network with branches extending deep into the canalicular structure, presumably connecting with osteocytes distant to the implant. For the first time, this study has shown that the interwined dendrites from osteocytes are also directly in contact with the implant surface (Fig.5). In addition to the intecellular connection between osteocytes via their cell processes, they were also connected with bone marrow through their dendrites (Fig 3). Interestingly, following the acid-etching process there were many discontinuous alternating regions of an undecalcified substance and dark areas that ran parallel the implant surface (Fig 6). While most of the osteocytes were situated beside this dark area, this region however was not totally devoid of osteocytes (Fig.7). This ‘dark area’ could be interpreted to mean that the implant was surrounded by a sparse, poorly calcified area which was easily removed by acid-etching. This hypothesis is supported by the SEM elemental scanning analysis, which showed that this area, nearly 6-7 μm wide, had gradually decreasing levels of calcium and phosphate ions as it approaches the implant surface (Fig.8).

Figure 4. Osteocyte situated in lacuna with its dendrites connected to others, forming LCN structure. (Right image is an amplified osteocyte image of the left box in right image).
Figure 5. Osteocyte (red arrow) with its dendrites in direct contact with implant surface (Right image is the amplified image of the box in left image).

Figure 6. (a) Low magnification shows alternating regions of an un-decalcified substance (yellow arrows) and dark gaps (orange arrows) alongside implant. (b) Higher magnification shows most of the osteocytes (red arrows) were shown to be alongside the dark gap adjacent to the implant after the acid-etching process.
Figure 7. (a) High magnification image shows osteocytes (red arrows) situated in the ‘dark area’ as described in Fig 6, that are in direct contact with implant. (b) In the alternate undecalcified substance areas as described in Fig 6, osteocytes (red arrows) were shown to be attached to the implant surface via this thin undecalcified substance (between yellow dashed lines) which is directly in contact with the implant.

Figure 8. SEM/Energy-dispersive spectrometer (SEM/EDS) elemental analysis of the mineralised tissue. (a) Shows the line scan position in relation to the implant (yellow line). (b) High magnification of the elemental line scan (~40 μm). (c) The Ca and P ion concentrations gradually decreased over a distance of 6-7 μm (red bars) towards the implant surface. This 6-7 μm area corresponds to the previously described ‘dark area’.
Figure. 9 Diagram (a) shows the implant embedded in bone tissue (blue zone) that consists of lower density mineralized tissue which represents the dark areas alternating with undecalcified substance). The dendrites derived from osteocytes are intertwined with the implant to reinforce osseointegration. The advantage of this type of structure is that it allows the distribution of masticatory forces and micro-movement of the implant. This zone acts like a cushion similar to that of the natural periodontal structure (b). (c) Enlarged diagram of area adjacent to implant (red box). Blue spots represent Ca ions and green spots represent P ions. Because of the sparse and poor mineralization and less organic tissue near the interface under acid-etching conditions, mineralized substance is removed quickly producing a relative dark area.

6.5 Discussion

Using a novel acid-etching methodology combined with SEM imaging, this study confirmed the hypothesis that osteocytes maintain close contact with the implant surface during osseointegration, demonstrated by the ultrastructural observation of osteocyte dendritic structures in direct contact with the implant surface.

Few studies have examined the relationship between osteocytes and the dental implant surface.[20, 21] Using classical histological observation, previous reports have focused on changes in osteocyte density around the dental implant to infer the role of osteocytes in osseointegration. Until now, little information has been published regarding the ultrastructural relationship between osteocytes and dental implants.
The most frequently used methods for analyzing the interface between cells (including osteocytes and their LCN structure) and/or tissues and implants following osseointegration have been traditional histology methods using light microscopy or scanning electron microscopy in the backscattered electron mode. [22] In order to examine the interface between bone tissue and the implant, it is preferable for the sections to include both the implant and bone tissue. To achieve this with current technologies, samples need to be embedded in resin, cut using a diamond saw and ground until the sections’ thickness is between 15-50μm. The histological sections used in this study were approximately 30μm thick and showed similar characteristics to other reported studies, [23, 24] i.e. the host bone was in close-knit contact with the implant when viewed at both low and high magnification. Unfortunately, given the thickness of the sections, it was difficult to visualize osteocytes, much less their LCN structure. Indeed, even with conventional paraffin embedded 5μm thick tissue sections, other reported studies have also shown that it is difficult to visualize the delicate LCN structure. [22] Using the BSE-SEM method, close contact between bone tissue and the implant surface was observed at lower magnifications. At higher magnifications, some areas exhibited a 2-2.5μm gap, which was most likely an artifact caused by shrinkage during the resin polymerization process. Notably, neither of these routine methods can differentiate the 3D ultrastructure of osteocytes around osseointegrated implants.

Bone has a hierarchical structure at both the microscopic and macroscopic level, which allows it to perform its primary function of providing support and distributing biomechanical load. After treating the samples with the acid-etching protocol, it was shown that the osteocytes situated in the resident bone away from the implant were arranged in an orderly fashion, which is indicative of adaptation to mechanical loading. However, within the newly formed bone adjacent to the implant, osteocytes appeared to have a disorderly arrangement. One possible explanation for this observation is that the newly formed bone lacks functional adaptation as it is yet to
The ultrastructure of osteocytes on osseointegration

undergo remodeling in response to loading stimuli. The different appearance of the osteocyte arrangement between mature and newly formed bone implies that osteocytes around implants may change orientation once exposed to the appropriate stimuli. However, further ultrastructural studies of osteocyte orientation around loaded implants are required to confirm this hypothesis.

There were also relatively more osteocytes in the newly formed bone, especially within the implant thread area, when compared with the original trabecular bone. This is consistent with a previous report which examined the relationship between osteocytes and implants.[21] In that study, a higher osteocyte density was observed near the implant after a relatively short time span (1-5 years) following surgery, which subsequently decreased with increasing time (14-27 years). In this respect, clearly one of the limitations of the present study is the use of a single time-point for investigation (8 weeks) as it is then impossible to predict any temporal changes in osteocyte numbers/density or morphology over longer periods of bone modeling and remodeling. As osseointegration is a dynamic process, it would be interesting to use the methodology described in this study to examine the temporal changes in osteocytes and their relationship with the implant surface.

Osteoblasts/osteocytes play a key role in the establishment and maintenance of osseointegration. Osteocytes residing in the lacuna, representing the terminally differentiated cell of the osteoblast lineage, were seen to be interconnected with each other by means of their dendritic cell processes. This network, named the lacuno-canalicular network (LCN),[25] is known to play a vital role in nutrient transport, cellular communication[26] and mineralization. The osteocyte LCN structure has been shown to have the ability to sense mechanical strain, and regulate adaptive responses by coordinating the bone-remodeling processes. [27, 28] Taken in the context of the contemporary literature about the function of osteocytes,[8, 29] the observations reported in this study, suggest that osteocytes situated in the LCN...
network regulate the dynamic bone metabolic processes that are necessary for maintaining long-term successful osseointegration.

According to the concept of osseointegration, the titanium implant is in direct contact with host bone. In previous in vitro models, it was noted that there was a layer of protein matrix between osteoblasts and the implant surface.[17, 18] However, due to methodological limitations, there has been a lack of definitive evidence in vivo to support the notion that osteoblasts/osteocytes come in direct contact with the implant surface.

Using a novel acid-etching method together with SEM imaging, this study clearly shows that osteocyte dendrite structures are in direct contact with the implant surface. This not only provides a deeper understanding of the osseointegration process, but also new opportunities to investigate the relationship between osteocytes and implants in health and disease. The results of the study suggest that during the early stages of bone formation, osteoblasts establish contact with the implant surface and maintain this relationship until they differentiate into osteocytes and become embedded in the mineralized matrix. Subsequently, the osteocytes utilise their dendrites to maintain direct contact with the implant and the LCN network, thus ensuring adequate nutritional supply and facilitating mechanosensing in the local microenvironment.

The response of bone tissue to dental implants depends on many factors including the implant surface, the time at which the sample was evaluated, the recipient site and the animal species used. Using a rat maxilla model similar to that used in the present study, Futami et al (2000)[30] showed that the establishment of osseointegration (at the light microscope level) occurred considerably earlier (4weeks post-implantation) than that reported in other studies (6-12 weeks post-implantation) which used endochondral recipient bones such as the femur, tibia and fibula.[31, 32] Therefore the eight-week healing time-point used in the present study suggests that the observed ultrastructural arrangement of the osteocytes and
their LCN may be representative of new bone that has undergone a period of remodeling.

Irrespective of whether osseointegration occurs via distance’ or ‘contact’ osteogenesis [33], given that the maxilla develops by intramembranous ossification, it is not surprising that intramembranous rather than endochondral ossification occurs around dental implants placed in the rat maxilla. *In vivo* studies in both animals and humans have demonstrated that the implant surface topography and or chemistry can also affect both the rate and degree of osseointegration.[23, 34]. While this study used a moderately rough titanium implant surface That is likely to favour ‘contact’ osteogenesis, whether surface characteristics that increase the degree of roughness at the micro and/or nano-scale would also influence the osteocyte- LCN morphology and/or the arrangement around the implant surface is unknown and requires further investigation.

Interestingly, many relatively dark areas were noted at the implant interface with alternating areas of a thin undecalcified substance. These dark areas approximately 6 - 7µm wide were not the same as the shrinkage gap following processing that varied in size up to a maximum distance of only 2-2.5µm wide. Given that the purpose of using the acid-etching protocol is to remove the mineralized tissue and keep non-mineralized and organic tissue intact, a dark area usually means that more mineralized tissue has been removed. However, in the time allotted for the acid etching process, this phenomenon presumably would only happen in a relative sparse and less mineralized tissue with a higher organic component. Using SEM elemental analysis these areas were indeed less mineralized, as the density of calcium and phosphorous ions gradually decreased as it approached the implant surface over an area that was nearly 6-7 µm in length. The dark area also may be a reflection of increased metabolism in the microenvironment around the implant. In addition to the dark areas representing reduced mineralization, undecalcified areas were also shown to exist around the implant. In these areas,
osteocyte contact with the implant surface was via a thin undecalcified substance. This is consistent with previous studies that have reported a thin undecalcified substance consisting of three different layers. The first is a 20-50nm collagen-free zone, followed by a 100-500nm zone of randomly distributed collagen fibers in which osteocytes were occasionally found. The third layer consisted of collagen fibers oriented in orderly bundles.[35]

Many other previous studies have also reported the presence of an intervening layer containing cellular elements and/or amorphous substances at the bone-implant interface. This non-mineralised amorphous layer on the implant surface most likely represents interposed cells that have been compressed and degenerated by either space constrictions, nutrient deficiency and/or compaction by new bone deposition during the remodeling process.[33, 36] While the function of this layer is as yet unresolved, the large number of fibroblast-like cells, multinucleated giant cells and macrophages present suggests it may ultimately serve as a means to make space for new bone formation.

The results of this study suggest that the implant is embedded in a bone tissue matrix consisting of both areas of decreased mineralization where osteocytes are in direct contact with the surface, and thin zones of undecalcified substance through which the osteocytes connect to the implant surface via dendritic processes. This structure may partly replicate the periodontal ligament microenvironment and proprioceptive function around natural teeth (Fig.9), albeit to a lesser extent, whereby the osteocyte dendritic processes provide a cushion around the implant that not only allows for small elastic micro-movements that are advantageous for the distribution of masticatory forces, but also provides a potential mechanism for osseoperception.

A further consideration regarding the process of osseointegration is the timing of implant placement following tooth extraction. From a number of systematic reviews of the literature, there is currently insufficient evidence that either
immediate or delayed implant placement results in improved osseointegration in the long term.[37, 38] The healing mechanism of the bone tissue may be expected to be the same regardless of the implant placement timing protocol, especially when there is an absence of a gap between the implant and the edges of the extraction socket, as was the case in this study. Therefore, it is unlikely that that the osteocyte-LCN morphology would be significantly affected by the implant placement timing, although this needs to be confirmed in additional studies.

6.6 Conclusions

Resin-casting of samples containing metallic implants and bone followed by phosphoric acid etching is an effective technique for visualizing the ultrastructure of osteocytes and the LCN around osseointegrated implants using SEM. Visualisation of the nature of the interaction of osteocyte dendrites with the implant surface allows us to better understand the ultrastructure of osseointegration and the micro-dynamic metabolic processes associated with implants in host bone. The finding of a low-density mineralized zone immediately adjacent to the implant along with osteocyte dendritic processes contacting the implant surface may explain the biomechanical mechanism through which implants can adapt to changing masticatory forces and thereby support prostheses for long periods of time. This study suggests that the osteocyte-LCN structure is likely to play an important role in maintaining the dynamic metabolic microenvironment around implants, with osteocytes having a vital role in maintaining successful long-term osseointegration.
6.7 References

17. Sammons, R.L., et al., Comparison of osteoblast spreading on microstructured dental implant surfaces and cell behaviour in an explant


Chapter 7: The effects of osteoporosis and implant topography on osseointegration in the rat maxilla
The effects of osteoporosis and implant topography on osseointegration in the rat maxilla.

Zhibin Du\textsuperscript{1}, Yin Xiao\textsuperscript{1}* , Saeed Hashim\textsuperscript{2}, Stephen Hamlet\textsuperscript{2}, Saso Ivanovski\textsuperscript{2}*

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\underline{Prof. Yin Xiao} \\

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7.1 Abstract

Compromised bone quality and/or healing in diseases such as osteoporosis are recognised risk factors for dental implant osseointegration. This study aimed to: 1) examine the effects of experimentally induced osteoporosis on implant osseointegration using a novel rodent model and 2) whether micro-rough implant surface topography could compensate for the any deleterious effects of estrogen deficiency.

Machined and micro-rough surfaced implants were placed into the first molar mesial root socket immediately after tooth extraction in the maxilla of ovariectomised (OVX) and sham-operated (SHAM) rats. The rats were sacrificed after 3, 7, 14, and 28 days post-implantation and osseointegration of the implant was evaluated by histological, scanning electron microscopy (SEM), and quantitative real-time PCR (qPCR) analysis.

Osseointegration as measured by the percentage of bone to implant contact (%BIC), was significantly higher around rough surfaced implants in the OVX animals when compared to machined implants in OVX animals at day 14. The %BIC in the OVX rough implant group was similar to that seen with both rough and smooth implants in SHAM animals at this time. By day 28 however, similar levels of %BIC were found for all test groups. This suggests the rough surface stimulated a more rapid osseointegration process in OVX animals.

To assess the possible molecular mechanism(s) responsible for this accelerated osseointegration on the rough surface, the level of gene expression of osteoblast, osteocyte, osteoclast and inflammatory markers at day 3 and 7 were examined. At day 3 the rough surface implant significantly increased the expression of alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen (COL1A), receptor activator of nuclear factor kappa-B ligand (RANKL), tartrate-resistant acid phosphatase (TRAP) and dentin matrix protein 1 (DMP1). By day 7, the expression of the inflammatory markers was lower as expected with resolution of the initial inflammation at implant placement. Expression of the bone markers increased further overall, although there were few significant differences in the levels of expression comparing the rough and machined surfaces at this time. These
molecular results correlate with the morphological (histology and SEM) observations at these early healing time points.

In conclusion, osteoporosis was found to reduce the early osseointegration (as measured by %BIC) of machined implants. However, the rough surfaced implant appeared to trigger a cell response able to compensate for the estrogen deficient conditions resulting in %BIC similar to that seen in SHAM animals. Nevertheless, this difference was not maintained as there was no significant difference in %BIC between the OVX and SHAM groups regardless of implant surface in the long-term (day 28).

Key words: osteoporosis, rat, maxilla, dental implant, osseointegration.
7.2 Background

Osteoporosis is a chronic disease affecting over 200 million people worldwide [1]. Osteoporosis in the elderly, especially postmenopausal women is also significantly correlated with tooth loss [2-5]. Lower bone density, poor bone quality and osseous microstructural changes, all characteristics of osteoporosis, have been shown to delay the bone healing process of fractured bone [6-8]. As endosseous implant healing has a similar mechanism to that in bone fracture healing [9], it is reasonable to assume that dental implant healing in osteoporosis patients may be negatively affected, however this is still a controversial issue [10-12]

Animal studies have reported a relatively lower rate of osseointegration in an osteoporotic environment, however it should be noted that the majority of these studies were based on the long bone [13-16] rather than jaw bone [17, 18]. While limited studies in the mandible using an ovariectomized (OVX) rat model showed no significant difference in bone formation around the implant [17, 19], these results have limited clinical significance, as it is the maxilla, especially in the posterior area, of osteoporotic patients where there is a high risk of dental implant failure because of its porous trabecular bone [20].

Implant design and topography are well-known factors to influence peri-implant tissue healing [21]. Most animal studies have shown that rough surfaced titanium implants result in superior bone to implant contact compared to machined implants [22, 23], as well as having superior torque removal values [24, 25]. However, there have been no studies showing whether these rough surfaced implants are able to compensate for the effects of osteoporosis on the implant healing process.

We have previously shown that the rat maxilla area is a good location to assess the effects of osteoporosis on alveolar bone quality [26], as well as for dental implant research [27]. In this study, the rat maxilla model was used to determine whether estrogen deficiency would affect osseointegration in the posterior maxilla and whether rough surfaced implant could partly compensate the negative effects on osseointegration due to osteoporosis.
7.3 Material and methods

Animals

The experimental protocol for the study was approved by the Griffith University animal ethics committee (DOH/01/4/AEC). Forty-six three-month-old female Sprague-Dawley rats (Murdoch, Western Australia) were used for the study. Animals were fed standard rat chow and water ad libitum throughout the experiment. After acclimatization for 2 weeks, the rats were randomly divided into two groups, sham-operated (SHAM, n=23) and ovariectomised (OVX, n=23). Ovariectomy was performed according to previously established methods [14]. SHAM group rats were also subjected to the same surgical procedure with an equivalent amount of fat tissue removed instead of the ovaries. All the operations were performed under isoflurane (1–3%) inhalation anaesthesia. The animals were subsequently allowed to develop osteoporosis over three months before implant placement. This period of time has been shown to be sufficient to develop osteoporosis in this model [26].

Implants

Minimally-rough ‘machined’ (Ra = 0.74 μm) and rough (Ra = 1.27μm) surfaced titanium implants (2 x 3mm) produced from Type IV commercially pure titanium were obtained from Southern Implants Ltd (Irene, South Africa). The rough surfaced implant was prepared using the same techniques as used for commercially available dental implants (Fig.1).
Surgical Procedures

Implant placement surgery was carried out 3 months after ovariectomy according to previously published procedures [27]. Briefly, implants were immediately placed into the first molar mesial socket after tooth extraction. The osteotomy was prepared using a 1.2mm pilot drill and a 1.8mm diameter final drill (Southern Implants, Ltd., Irene, South Africa). All osteotomy procedures were performed with copious saline irrigation. Machined and rough surfaced implants were placed into the prepared sockets with a torque of 15-20Ncm until the implant coronal plane was at the level of the bone crest. The wound was then sutured with resorbable sutures (5/0 Vicryl). Post-operative analgesia (buprenorphine 0.01 - 0.05mg/kg and carprofen 4 - 5mg/kg) and antibiotic cover (entrofloxacin 2.5mg/Kg) were administrated by intraperitoneal injection immediately after the surgery and continued daily for three days post-operatively.

To assess subsequent new bone formation, fluorescent dyes; alizarin red S (25mg/kg, 3 days before sacrifice) and calcein (10mg/kg, 10 days prior to alizarin injection) were given intraperitoneally. Animals were sacrificed after 3, 7, 14 and 28 days of healing and samples were collected for histological, SEM and qPCR analysis.

Histological Sample Preparation

Using a diamond-tipped circular saw, block sections of the maxilla containing the implants were collected and immediately fixed with 4% paraformaldehyde for 48h at 4°C. The samples were then dehydrated in a graded series of ethanol before embedding in methylmetacrylate resin (Technovit® 7200 VLC, Heraeus Kulzer, Dormagen, Germany).

Thirty-micron thick sections of the maxilla containing the implant were prepared using a cutting and grinding system (EXAKT Apparatebau, Norderstedt, Germany) [28]. The sections were stained with methylene blue-alizarin red S and scanned using the Aperio ScanScope CS at 40 times magnification (Aperio Technologies Inc., Vista, CA).

Histomorphometric analysis was carried out using ImageScope™ software at 4 times magnification (Aperio Technologies Inc.). The percentage of bone to implant contact (%BIC) is defined as the ratio of the sum of the implant surface in direct contact with new bone tissue.
and the total length of the implant adjacent to native bone (each side contained at least two threads). The new bone area (%BA) is defined as the percentage of mineralized bone tissue within the threaded areas of the implant that were adjacent to native bone [27].

**Scanning electron microscopy**

The cryo-fracture technique [29], was used to prepare osseointegrated samples for SEM observation (Carl Zeiss Sigma VP Oxford Micro-analysis Field Emission Scanning Electron Microscopy (FE-SEM), Germany). Samples (n=2 in each group) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (PH 7.4) containing 0.05% tannic acid for at least 48h at 4°C. The samples were then frozen in liquid nitrogen, followed by the cryo-fracturing.

**Real Time Quantitative PCR**

After unscrewing the implant from the maxilla, tissue from the implant surface and socket wall was collected using small dental curette and immediately immersed into TRIZOL reagent (Invitrogen). Total RNA was subsequently extracted from these samples according to the manufacturer’s instructions. M-MuLV Reverse Transcriptase was then used to prepare complementary DNA (cDNA) according to the manufacture’s protocols.

Oligonucleotide primers used to assess osteogenic (alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen (COL1A)), osteoclast (receptor activator of nuclear factor kappa-B ligand (RANKL), tartrate-resistant acid phosphatase (TRAP)), osteocyte (dentin matrix protein 1 (DMP1), sclerostin (SOST)) and inflammatory marker gene expression (tumor necrosis factor-alpha (TNFα), interleukin-1β (IL-1β)) are described in table 1. Real-time PCR reactions (LightCycler 480 Real-Time PCR System, Roche, USA) were run using the following parameters: denaturation at 95°C for 30 s and 40 cycles of amplification (95 °C for 10 s and 60 °C for 20 s). Quantification of gene expression was calculated using the delta Ct method and 90% PCR efficiency (k*1.9Δct) [30]. The expression of the targeted genes were normalised using the geometric average of three house-keeping genes (B2M, GAPDH and β-actin) [31].
Statistical analysis

One-Way ANOVA followed by Tukey’s multiple comparison tests were used to compare differences in the mean %BIC, %BA, %MAR and relative gene expression between the implant surfaces (machined vs rough) and within each treatment group (SHAM vs OVX) at all-time points. A p-value of <0.05 was considered significant.
7.4 Results

Analysis of early healing.

After 3 days of healing, the cryo-fractured samples showed that more tissue was attached to the rough surfaced implants compared with the machined implants in both the SHAM and OVX groups (Fig. 2a, d; Fig. 3a, d). At high magnification, the rough surfaced implants were seen to be covered by a layer of mixed tissue including matrix, fibres and cells (blood and inflammatory cells) (Fig. 2b; Fig. 3b). However, on the machined implants, most of the surface was exposed or covered with a very thin mixed structure consisting of fibres and scattered cells with less amorphous matrix (Fig. 2e; Fig. 3e).

![Fig. 2](image)

**Fig. 2** Cryo-fracture SEM and histology images of implants after 3 days of healing in the OVX group. The moderately-rough surfaced implant showed more adherent mixed tissue compared to the smooth implant (a, b vs d, e), the attached mixed tissue include fibres, cells and amorphous matrix (b). The smooth implant was covered with a thinner layer of tissue with clear fibres and cells (e).

Histological analysis showed the moderately-rough surfaced implant had osteoblasts organised near the old bone surface ((c), yellow arrows) compared with the smooth surface (f). ((c, f) bar=80μm).

The morphology of the tissue covering the implant surfaces (rough & machined) in the SHAM group revealed a denser quality of fibres compared with the OVX group (Fig. 3b vs Fig. 2b; Fig. 3e vs Fig. 2e). Inflammatory cells were visible on the surface of the implants in both the OVX and SHAM groups at this early stage of healing (Day 3).
Histological analysis revealed a small amount of early osseointegration in SHAM treated animals with rough surfaced implants, with a thin layer of new bone seen to be forming directly on the implant surface (Fig.3c). However, overall there was no obvious difference between the SHAM and OVX group for both rough and machined implants at this early time-point (Fig. 2c, f; Fig. 3c, g).

Fig. 3 Cryo-fracture SEM and histology images of implants after 3 days of healing in the SHAM group. The moderately-rough surfaced implant again had more adherent tissue than the smooth surface (a, b vs d, e), which is consistent with the OVX results (Fig. 2). However, the tissue’s quality, especially the fibres in the SHAM group appears to be better than the OVX group (Fig. 3b vs Fig. 2b; Fig. 3e vs Fig. 2e). Inflammatory cells are easily visible at this stage ((e), purple arrow). Histological images show the earliest osseointegration was seen in the SHAM moderately-rough surface implant ((d), orange arrow). (c, f, bar=80μm)

The host bone was in contact with the implants in some areas, with fragments of old bone also sparsely distributed within the implant thread chambers. Osteoblasts were found to be present at this stage and were more obvious on the rough surfaced implants, especially in OVX animals, which showed osteoblasts regularly organized near the old bone surface (Fig. 2c).

Confocal fluorescence analysis at day 3 showed there was scattered new bone formation in the implant chamber areas as shown by the uptake of calcein fluorescence by the new bone
(Fig. 4). Furthermore, the SHAM group were found to have more new bone formation than the OVX group, while rough surfaced implants in the OVX group have better new bone formation than machined surfaced implants in OVX animals (Fig. 4).

**Fig. 4** Calcein and Alizarin red staining of new bone formation. Confocal imaging revealed new bone formation in SHAM group implants and OVX rough surface implant (green fluorescence ). However, compared with the SHAM group, new bone formed in the OVX group is limited, which indicates early osteogenesis could be inhibited by ovariectomy. OVX smooth surface implants showed almost no new bone formation at this stage. (Bar=50μm)

By day 7 of healing, under SEM observation, the rough surfaced implants were again found to have more mixed tissue than the machined implants. The tissue attached to the implant surfaces also seemed denser than it appeared at day 3 [Fig. 5a, d; Fig. 6a, e]. Under high magnification, fibers merged with each other to become thicker compared with their morphology at day 3 in both the SHAM and OVX groups. The fiber distribution in the SHAM group implants was thicker and more regular than the OVX group implants [Fig. 6b vs Fig. 5b; Fig. 6f vs Fig. 5e]. The rough surfaced implant group also showed more attached fibers than the machined implant group [Fig. 5b vs 5e; Fig. 6b vs 6f]. Osteoclast-like cells could be readily observed among the tissues associated with the OVX rough surfaced...
implants [Fig. 5b]. Blood and inflammatory cells decreased compared with day 3 but still can be observed especially on the OVX machined implants [Fig. 5e].

Histology showed that new woven bone was formed around both machined and rough surfaced implants. The SHAM group formed more bone to implant contact than the OVX group (Fig. 6c vs Fig. 5c; Fig. 6g vs Fig. 5f), and the rough surfaced implant group showed more osseointegration than the machined surfaced implant group (Fig. 5c vs 5f; Fig. 6c vs 6g). The new bone formed in the SHAM rough surfaced implant group seemed more mature than in either the SHAM machined or both OVX groups (Fig. 5c). New bone formation was also observed at the junction of the old bone surface in all groups. Both osteoblast and osteoclast activity can be observed, with osteoblasts arranged regularly along the new bone surface (Fig. 6d), and multinuclear osteoclasts located in the old bone surface within obvious bone resorption pits (Fig. 6h).
Fig. 6  Cryo-fracture SEM and histology images of day 7 results in the SHAM group. More tissue was attached to the Rough surface than the smooth surface implant. SHAM rough surface showed attached regular merged fibres, with less mixed cells than smooth surface implant (Fig. a, b vs e, f). The SHAM group attached tissue clearly showed better quality than OVX group (Fig. 6b vs Fig.5b; Fig. 6f vs Fig. 5e ). Histology images showed the SHAM rough surface implant not only has more osseointegration than smooth surface implant, but also has the most mature bone compared with SHAM smooth implant (g) and OVX group implants (Fig. 5c, f). Both osteoblasts and osteoclasts are visible. (d, h, bar=80μm; d, h, bar= 60 μm. Yellow arrows show the new bone quality, orange arrows show the osteoblasts and blue arrows show the osteoclasts )

Gene expression.

Two time points (day 3 and day 7) were selected for gene expression analysis of the early healing events. Gene markers were divided into four groups: osteogenic (ALP, OC and COL-1A), osteoclast (RANKL and TRAP), osteocyte (DMP1 and SOST) and inflammation (TNFa and IL1b). At day 3, overall low levels of osteogenic (ALP, OC and COL1A), osteoclast (TRAP) and osteocyte (DMP1) gene expression was shown to be significantly higher on the SHAM rough surfaced implant when compared to the other groups. Osteoclast (RANKL) gene expression was also significantly increased in the OVX rough surfaced implant group (Fig. 7) Furthermore, expression of the osteocyte marker DMP1 was significantly increased in both SHAM and OVX rough surfaced implant group when compared with the machined implant groups.
By day 7, gene expression of ALP, OC, COL-1, RANKL, TRAP, DMP1 and SOST were all significantly increased in all groups when compared with the day 3 results. The osteogenic marker COL1A was significantly higher in the SHAM groups compared with the OVX groups. The osteoclast markers RANKL and TRAP were significantly increased in the SHAM rough surfaced implant group compared with other groups (Fig 7). Most noticeably, it is the TRAP rather than RANKL expression in the OVX rough surfaced implant group that showed significantly increased levels when compared with the corresponding day 3 result. The osteocyte markers, DMP1 and SOST also showed a trend towards increased levels at this stage. The inflammation markers IL1b and TNFa at 7 days showed decreased expression than at day 3, although this was not statistically significant [Fig. 7].

Fig. 7 Gene expression analysis of early bone and inflammatory markers. At day 3, ALP, OC, COL1A ,TNFa, DMP1 and TRAP were significantly increased in the SHAM rough surface implant. RANKL and DMP1 were also significantly increased in the OVX rough surface implant. At day 7, COL1A gene expression was significantly increased in SHAM group than OVX group. At the same time
point, the SHAM rough surface implant showed significant increase in RANKL, TRAP compared with other groups.

Analysis of late healing.

Histological analysis was also performed on the day 14 and day 28 samples. At day 14, there was a significant amount of new bone around all implants for both SHAM and OVX groups, with active osteoclasts and osteoblasts visible. The osteoblasts were arranged regularly around the new bone surface, while in some areas of the old bone, multinuclear osteoclasts could be observed in resorption pits (Fig. 8). There was no difference in %BIC between the SHAM rough surfaced implant group and the SHAM machined implant group. In addition, no difference in the %BIC was found between the SHAM rough surfaced implant group and the OVX rough surfaced implant group (Fig. 9c, f). The OVX machined implant group had the least osseointegration as measured by %BIC, when compared with the OVX rough surfaced implant group and SHAM machined implant group. However, there were no significant differences in %BA among the groups (Fig. 8, Fig. 9c, f).

Fig. 8 Histology results at day 14. New bone was formed around the implant in all groups. SHAM group implant and OVX rough surface implant showed more osseointegration than the OVX smooth surface implant (Fig. 9c). Both Osteoblasts (yellow arrows) and osteoclasts (blue arrows) are active at this point.
stage. However, the OVX smooth surface implant shows more osteoclasts (a, b, d, e, bar=100μm; c, f, bar=60 μm)

Fig. 9 Histology results at day 28. The new bone appears more mature than at day 14 (Fig. 8). Active osteoblasts can still be seen on the new bone surface; however, osteoclast numbers appear to be lower compared with the day 14 results. While %BIC was significantly lower in the OVX smooth group at day 14, the effect of OVX was not apparent in the rough group which was similar to that seen for the SHAM groups. By day 28, there were few significant differences in either %BIC or %BA between all the groups (c, f) (a, b, d, e, bar=100μm)

At day 28, the new bone formed around the implants had a more mature and dense morphology than at day 14. The %BIC for all groups increased, however only the OVX groups (rough and machined) were statistically higher compared to day 14.

Comparing %BIC in the rough and machined groups (SHAM c.f. OVX) there were no significant differences at this time. Furthermore, there was also no significant difference between the SHAM and OVX groups (rough c.f. machined). The %BA values also showed a similar trend increasing at day 28 compared with day 14 which was statistically significant for SHAM rough surfaced implants. Furthermore the %BA in the SHAM rough surfaced implant group was significantly higher compared with the OVX rough surfaced implant
group at this time (Fig. 9). Osteoblasts were still visible around the new bone surface however the number appeared fewer than at day 14. The mineralized apposition rate (MAR) as determined by fluorescence staining did not show any significant differences among the groups (Fig. 10).

![Fig 10. Calcein and Alizarin red staining of new bone formation at day 28 (a,b). There was no difference in the mineralized apposition rate (MAR) between groups (c).](image)

### 7.5 Discussion

The effect of osteoporosis on the osseointegration of titanium dental implants continues to be a controversial issue [10, 11]. As most osteoporotic animal models have utilized long bones, such as the tibia [12, 14, 16, 32, 33] or femur [15, 34], the results from these studies is of limited value in understanding the osseointegration of endosseous implants into the jaw in osteoporotic conditions. Compared with long bone, the jaw bone has a different embryological origin and a different ossification mechanism as well as a different response to estrogen deficiency [35, 36]. Thus, results from animal models of osteoporosis using the maxilla would be more comparable to implant healing in osteoporotic patients. The results of this study show that both OVX and implant surface morphology affected the early healing events around the titanium dental implants.

New bone formation was observed to be scattered in the implant thread chamber areas in the SHAM (rough and machined) and OVX rough surfaced implant groups as identified by calcein uptake as early as day 3 post-implantation. New bone on the implant surface was also
observed in the SHAM rough surfaced implant at day 3. These findings are consistent with those reported by Schwarz et al (2007) showing bone formation as early as the 4th day post implant insertion [37]. It is noteworthy that these workers used a dog model rather than the rat which when compared with the dog, has a faster metabolism and hence accelerated healing [38]. Although the earliest histological evidence of osseointegration was seen on the SHAM rough surfaced implant, it is difficult to conclude that the rough surfaced implant had the best osteoinductive/osteoconductive characteristics due to the limited sample size for histological analysis (n=2). Nevertheless, if we consider the histology results together with the cryo-facture analysis, which showed that the rough surfaced implants had more attached tissue than the machined implants, it is not unexpected that the rough surfaced implant should subsequently have accelerated bone formation compared to the machined implant. This is also consistent with previous findings that osseointegration occurs earlier on micro-rough surfaces via a process of ‘contact osteogenesis’ directly on the implant, while only ‘distance osteogenesis’ originating from the borders of the surgically prepared recipient bone bed is observed with machined surfaces [39].

Previous studies in vitro have shown that surface topography can affect cellular recruitment [40] and promote osteoblast differentiation in vitro [41]. The rough surfaced implant has been shown to increase osteoblastic cell attachment [42-44] and result in higher osteogenic gene expression (ALP, bone sialoprotein, OC) compared with machined implants [43]. Our study has also shown a significant increase in the expression of osteoblast markers (ALP, OC and COL1A) in the SHAM rough surfaced implant group consistent with previously reported data [43], which provides further evidence that the rough surfaced implant has a higher bone forming capacity than machined implants. However, the rough surfaced implant in the OVX group did not show an increase in osteogenic gene expression at this stage despite an increase in the number and organization of osteoblasts shown histologically on the OVX rough surfaced implant (compared to OVX machined group), which may indicate that estrogen deficiency may interfere with the bone forming process.
Bone remodeling is a coordinated process triggered by osteoblast and osteoclast activity. Osteoclasts also play an important role in the initial period after implant placement [45, 46]. Osteoclast activation by rough titanium surfaces in vitro has been shown to be similar to that of bone in vivo whereas activation is reduced by smooth titanium [47]. Furthermore, studies have shown that the gene expression of bone formation makers such as ALP, OC and collagen 1 were coupled with the increased expression of osteoclast markers [48, 49]. Compared with the machined implants, our data showed that the SHAM rough surfaced implant group not only significantly increased the expression of bone formation markers such as ALP, OC and COL1A at day 3, but also significantly increased TRAP gene expression, which is consistent with these previous reports [48, 49]. Interestingly in the OVX rough group, the gene expression of RANKL rather than TRAP significantly increased at day 3. The higher expression of RANKL could promote osteoclasts maturation, differentiation and activation through the activated RANK\RANKL\OPG pathway [50, 51]. However expression of the bone formation markers did not increase suggesting the estrogen deficiency may affect the balance between osteoblast and osteoclast activity in the early (day 3) healing process.

By day 7, apart from the inflammation markers which decreased as would be expected with the resolution of inflammation during healing, the gene expression of osteoblast, osteoclast and osteocyte markers were significantly elevated compared with day 3 and consistent with previous reports [48]. The increase in gene expression is also consistent with the histological and SEM observations, which showed more bone formation around the implants, as well as more mature attached tissue.

Comparing groups at day 7, the SHAM rough surfaced implants showed superior osteogenic capacity compared to the other groups, with gene increased expression of COL1A, RANKL and TRAP supported by histological and SEM observations of increased bone formation and tissue maturation. OVX negatively influenced osteogenesis as COL1A expression in the OVX groups was found to be significantly lower than in the SHAM groups, which is consistent with previous clinical findings that showed that high risk osteoporotic fracture patients had lower levels of COL1A expression [54, 55]. The rough surfaced implants...
showed some differences compared with the machined implants in the OVX group, with TRAP gene expression significantly increased. Although the TRAP expression was lower compared to the SHAM rough surfaced implants at this time point, but it reached a similar expression level to SHAM machined implants. The results suggest that bone metabolism was enhanced around the rough surfaced compared to the machined implants in the OVX group. The gene expression analysis was supported by both the SEM cryo-fracture and histologic assessment, which showed more mature tissue attached on the implant surface and more osseointegration formed around the micro-rough compared with machined implants. It is noteworthy that it is difficult to compare the %BIC between the different groups at the 7 day timepoint because the new bone formation is still very limited.

Recently Osteocytes have been shown to play an important role in the bone remodelling process, regulating both osteoblast and osteoclast activity [52, 53]. For example, osteocytes may regulate osteoblast activity through the Wnt/β-catenin pathway [54] and also regulate osteoclast activities through the RANK\RANKL\OPG pathway [55]. Osteocytes also can react to hormonal changes (such as estrogen deficiency), by increasing osteocytic apoptosis to trigger bone remodelling and resorption [56]. Targeted ablation of osteocytes has been shown to lead to osteoporotic conditions [57]. Our previous work has shown that osteocytes have a close ultrastructural relationship with dental implants [58], however, the potential role of osteocytes in osseointegration under osteoporotic conditions is still not clear.

The role of osteocytes in the establishment of osseointegration was also assessed. The key molecule DMP1 which controls osteocyte formation and phosphate homeostasis [59] was significantly increased on the rough surfaced implant at day 3 in both OVX and SHAM groups, which indicated that the rough surfaced implant may trigger an earlier osteocyte response than the machined implant, thus regulating both osteoblast and osteoclast activities. As mentioned earlier, the RANKL expression at day 3 was significantly increased in the OVX rough surfaced implant group. Recent research has shown that it is osteocytes rather than osteoblast that are the mainly source of RANKL [60, 61]. The increased RANKL and DMP1 gene expression in the OVX group indicated the the rough surfaced implants triggered
an earlier osteocytes response/bone remodeling process than machined implants, even under the influence of estrogen deficiency. The advantages of the rough surfaced implant were also supported by the SEM and histology observation.

At day 7, both DMP1 and SOST gene expression were significant increased compared with day 3 which also coresponded with increased osteoblast and osteoclast gene markers, which indicated osteocytes regulating bone formation were in an active state. The SOST gene, which is a negative regulator of osteoblast function as an antagonist of the Wnt/β-catenin pathway, was also highly expressed compared with the day 3 results. The gradually increase of SOST gene expression form day 3 to day 7 suggests a transition of the osteocytes’ regulatory role, which at the earlier timepoint (day 3) predominantly promotes bone formation, and then gradually evolves into a role that balances osteoblast and osteoclast activities.

By day 14, estrogen deficiency (OVX) negatively affected the osseointegration of the machined implants as indicated by the low %BIC. Osseointegration of the rough surface implants however was relatively unaffected with the %BIC similar to that seen in the SHAM group, suggesting that the rough surface largely compensated for the negative effect of OVX.

By day 28, there were no significant differences among the groups suggesting that sufficient healing time may also compensate for the disadvantageous effects of machined surface topography or estrogen deficiency. Our results are highly consistent with another report which also compared three different surfaces (two different commercial rough surfaces c.f. a smooth surface implant) and showed significant differences only in the early healing time points (2 and 4 weeks). After 6 weeks of healing, there were no significant differences among the groups [62]. The similarity in the degree of osseointegration between the OVX and SHAM groups at this late time-point (28 days) is in agreement with previous reports which show no significant difference in long-term implant survival rate between rough and smooth surfaced implants [63] or between osteoporosis and healthy subjects [12, 64, 65].
Moreover, the bone area (BA) and mineral apposition rates (MAR) did not show any significant difference among the two groups. These findings suggest that osteoporosis may primarily affect the interface areas of the implant.

7.6 Conclusion

Both ovariectomy and implant morphology affected the osseointegration of implants in the early healing stage. Osteoporosis delayed early bone healing around machined surface implant. However, the rough surfaced implants could compensate for the negative effect(s) of ovariectomy by triggering an early osteocytes/osteogenic response and thus facilitating the earlier osseointegration of rough implants.
7.7 References


Chapter 8: Conclusions and Recommendations
8.1 Summary of Research Outcomes

Osseointegration has been defined as a direct bone-to-implant contact without interposed soft tissue. A rigid, functional fixation of dental implants within the host bone site achieved by osseointegration is necessary to provide long-term anchorage with the ability to support masticatory function. The osseointegration process and consequent clinical success of dental implants becomes more challenging in compromised clinical situations like osteoporosis. However, researchers still do not fully understand the biological mechanisms involved in osseointegration, and the consequential effects of conditions like osteoporosis on this process remains obscure.

There are a lot of factors that influence osseointegration, amongst which systemic factors are considered to play an integral role. The implant and its host bone are the two major interacting entities, and minor changes to either entity would affect the process of bone formation around an implant. It is recognised that compromised bone negatively affect osseointegration, while a micro-rough implant surface results in superior osseointegration compared with a smooth surface. Many diseases and conditions may affect bone quality. These include disorders of bone mineral homeostasis or imbalance of bone remodelling diseases such as osteoporosis, diabetes mellitus, hyperparathyroidism, Cushing’s disease, Paget’s disease, and collagen disorders such as osteogenesis imperfecta, Marfan syndrome, and drugs affecting bone quality such as glucocorticoids, chemotherapeutic agents and bisphosphonates, among others [1]. Of these diseases, osteoporosis is the most prevalent worldwide; it is estimated that over 200 million people worldwide have osteoporosis[2]. In Australia, the effect of this disease causes fractures in 50% of women and 33.33% of men beyond the age of 60 years [3]. Osteoporosis causes reduced bone formation, leading to fractures and impaired bone healing. The relationship between osteoporosis and bone formation around implants is still unclear. The consensus of the ITI (International Team of Oral Implantology) is that there is only a weak association with the risk of implant failure, however, this conclusion was based on only two-case control studies[4].
Osteoporosis is a condition characterised by decreased bone mineral density and a deterioration of bone microarchitecture, resulting in compromised bone quality. Therefore, osteoporosis is an interesting model to study the effect of compromised bone quality on osseointegration. The ovariectomized rat is the most useful model to simulate human postmenopausal osteoporosis, and has been selected for this study. Existing literature remains inconclusive whether the jaw bone (maxilla) is affected by osteoporosis, and the clarification of this controversial point was one of the aims of this project.

Published information regarding the use of rat jawbones for dental implant osseointegration research is also limited and inconsistent. The recent consensus statement from the Eighth European workshop on preclinical in vivo research (ARRIVE guidelines) concluded rodents were primarily used for osseointegration research when examining the effects of systemic disease. However, when healthy animals were used, the predominant implant sites were extra-oral, i.e. tibia and femur [5]. There is an urgent need to assess the suitability and feasibility of placing dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model. Dental implant success relies on the process of osseointegration whereby the titanium is bound to bone tissue without any intervening soft tissue.[6] However the biological mechanisms responsible have not been fully elucidated, especially with regard to the possible role of osteocytes in the maintenance of osseointegration.[7] Based on the traditional methods such as light microscopy (LM), Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) it is difficult to observe the ultrastructural of relationship between osteocytes and dental implants following osseointegration. A new method which can visualize the ultrastructure of the implant-bone interface in three-dimensions (3D) would thus be beneficial in facilitating the study of the relationship between bone cells and the implant surface.

Additionally, the design, chemical composition and topography of the implant surface is known to influence peri-implant tissue healing and subsequent osseointegration [8], but whether a moderately rough surface compared to a smooth surface can overcome the
disadvantages resulting from osteoporotic conditions warrents further study. Given that implant therapy has a very high success rate in healthy patients with good bone quality and quantity, one of the major foci of current research is the performance of implants in compromised sites and patients. Therefore, understanding the extent to which moderately rough implants can overcome the negative influence of systemic conditions has a clear clinical relevance.

Based on above, the first part of the project explored whether a rat’s maxilla could be used as an appropriate model for osteoporosis. In order to obtain a comprehensive understanding of maxilla changes after ovariectomy, 3 dimensional Micro CT, together with histological analysis, was used to analyse the changes in the maxilla, and the observation time points were set at 8, 12, 16 and 20 weeks. However, this is very difficult to implement because of the irregular shape of maxilla and tooth roots, as well as the presence of the sinus and nose cavities. The furcation area, which consists entirely of trabecular bone, is the most appropriate site to reflect the impact of osteoporosis on bone quality. Therefore, the furcation areas of the first molar and second molars were selected for micro CT analysis, assuming that any changes to the trabecular bone in the area would be representative of changes in the remainder of the maxilla. Significant changes were observed in the rat maxillae as early as 12 weeks following surgery. The results also confirmed that estrogen deficiency affects the jawbone resulting in compromised bone quality, thus establishing the rat maxilla as an appropriate model to explore the bone–implant healing process in osteoporosis.

In order to assess the effects of various parameters (such as osteoporosis and implant topography) on osseointegration and bone regeneration, various animal models have been routinely used before proceeding to human clinical trials. Small animals such as rodents (mice, rats, rabbits) are often used before moving to larger animal (dogs, sheep, pigs, primates) studies. However, published information regarding the use of rat jawbones for dental implant osseointegration research is also limited and inconsistent. The second part of the study assessed the suitability and feasibility of placing dental implants into the rat maxilla and to establish parameters to be used for dental implant research using this model. Forty two
customised titanium implants (2×3mm) were placed bilaterally in the maxillary first molar area of 21 Sprague-Dawley rats. Resin-embedded sections of the implant and surrounding maxilla were prepared for histological and histomorphometric analyses. The results showed the mesial root of the first molar in the rat maxilla was the optimal site to place the implant. 28 days of healing can be considered as an appropriate end point to assess the degree of osseointegration as measured by percentage bone-to-implant contact (%BIC) and bone area (%BA). The recommended implant dimensions for this particular site in the rat is 1.5mm in width and 2mm in length.

Recently, mounting evidence supports a prominent and multi-factorial role for osteocytes in bone homeostasis, such as as a mechanosensor perceiving strain signals through its dendritic processes or the bending of cilia [9-11], reacting to hormonal changes such as estrogen deficiency [12] and regulating mineralization [13] etc. The third part of this study tried to investigate the feasibility of using the resin-cast osteocyte method to observe the relationship between the LCN (including osteocytes) structure and the implant surface at the ultrastructural level in osseointegrated implant samples. The resin embedded samples were etched with 37% phosphoric acid and sections were coated with gold and examined using a Carl Zeiss SIGMA VP field emission scanning microscope. The results showed the osteocytes to be less organized in the newly formed bone around the titanium implant. Further, a layer of mineralization with less organic components was observed on the implant surface. This study has shown for the first time that osteocytes and their dendrites were directly connected with the implant surface and suggested an important regulatory role for osteocytes and their lacunar–canalicular network in maintaining long term osseointegration.

The final part of the study was been designed to assess the effects of osteoporosis on osseointegration and whether rough surfaced implants could have some potential to overcome the negative effects on osseointegration due to osteoporosis. The osseointegration process was evaluated by both histological and scanning electron microscopy (SEM) analysis. The molecular effects of estrogen deficiency on cells involved in the osseointegration process was also examined by assessing the expression of bone remodelling markers using quantitative
real-time PCR (qPCR). This included bone formation markers such as alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen (COL1A), osteoclast markers such as receptor activator of nuclear factor kappa-B ligand (RANKL) and tartrate-resistant acid phosphatase (TRAP), osteocyte markers such as dentin matrix protein 1 (DMP1), and sclerostin (SOST), and inflammatory marker gene expression tumor necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β)).

Osseointegration as measured by the percentage of bone to implant contact (%BIC), was significantly higher around rough surfaced implants in the OVX animals when compared to machined implants in OVX animals at DAY 14. The %BIC in the OVX rough implant group was similar to that seen with both rough and smooth implants in SHAM animals at this time. By day 28 however, similar levels of %BIC were similar for all test groups. This suggests the rough surface stimulated a more rapid osseointegration process in OVX animals.

To assess the possible molecular mechanism(s) responsible for this accelerated osseointegration on the rough surface, the level of gene expression of osteoblast, osteocyte, osteoclast and inflammatory markers at day 3 and 7 were examined. At day 3 the rough surface implant significantly increased the expression of alkaline phosphatase (ALP), osteocalcin (OC), alpha-1 type I collagen (COL1A), receptor activator of nuclear factor kappa-B ligand (RANKL), tartrate-resistant acid phosphatase (TRAP) and dentin matrix protein 1 (DMP1). By day 7, the expression of the inflammatory markers was lower as expected with resolution of the initial inflammation at implant placement. Expression of the bone markers increased further overall, although there were few significant differences in the levels of expression comparing the rough and machined surfaces at this time. These molecular results correlate with the morphological (histology and SEM) observations at these early healing time points. OVX also reduced the osteocyte’s body size rather than its lacuna.

In conclusion, osteoporosis was found to reduce the early osseointegration (as measured by %BIC) of machined implants. However, the rough surfaced implant appeared to trigger a cell response able to compensate for the estrogen deficient conditions resulting in %BIC similar to that seen in SHAM animals. Nevertheless, this difference was not maintained as
there was no significant difference in %BIC between the OVX and SHAM groups regardless of implant surface in the long-term (day 28).

8.2 Major Contributions

This project observed the effects of osteoporosis and implant morphology on the osseointegration. The ovariectomised rat is a frequently used model for osteoporosis as recommended by the U.S. Food and Drug Administration (FDA) [14]. However, whether the rat’s maxillary and/or mandibular bone can be used to study osteoporotic bone loss compared with the routinely used long bones or vertebrae is still a controversial issue [15] due to the different endochondral and membranous bone formation between long bones and maxillar/mandibular bones [16]. The maxilla is an irregularly shaped bone due to the presence of the sinus and nasal cavity in the molar area, which makes the micro CT analysis challenging. We created a novel method to analyse the representative furcation area among the roots to resolve this issue perfectly.

How to place the implant into maxilla was also a challenge for the project. Information about implant placement into the maxilla is confused with insufficient data reported in the literature. We found the mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research.

Osseointegration is a key word for this project. However knowledge about osseointegration has been mainly acquired from microscopic observation. This research developed a novel methodology to visual the 3-D relationship of osteocytes and the implant which will benefit the future research on the potential role of osteocytes in osseointegration.

Based on the above animal model platform, we further observed the effects of osteoporosis and implant surface topography on osseointegration. We found estrogen deficiency only affected the early implant healing and the implant morphology also affected the implant early healing process.

Briefly, the major findings of the thesis are as follows:
We created a new method to estimate the irregular maxilla bone density changes under estrogen deficiency.

We demonstrated the mesial root socket of rat maxilla can be used a model for dental implant research.

We created a novel method to observe the 3-D relationship between osteocytes and the implant surface.

We showed estrogen deficiency only affected the implant early healing period and the rough surfaced implant can overcome this negative effect.

8.3 Limitations & Recommendations for Future Work

The first part of our research proved the rat’s maxilla is a good model for osteoporosis research. This conclusion was based on the 3-D micro CT analysis. However, this method was based on an analysis of the furcation area only. While we believe that this area is representative of the whole maxilla, analysis of whole maxilla is difficult because of the technology involved. Further work is therefore needed to find a new method to analyse the whole maxilla rather than just one area.

In the second part, we found the mesial root socket of the first molar in the rat maxilla is a useful model for dental implant research. However, the implant placement operation requires a high degree of skill which is a limiting factor. Furthermore the implant used for this model is only suitable for small animal use. Compared with the generally used commercial implant for humans, this is a clear limitation of the study. Future work in a large osteoporotic animal model using commercial implants is also required.

In third part of our research, we found osteocytes had a close relationship with the dental implant. In terms of the multi-factorial role for osteocytes in bone homeostasis, osteocytes may react to hormonal changes such as estrogen deficiency, which through an increase in osteocyte apoptosis, triggers bone remodeling and resorption. Osteocytes may also play a role in mineral (phosphate) regulation and biomineralization via the action of osteocyte-specific proteins such as PHEX, DMP-1, MEPE, and FGF-23. It has also been shown that osteocytes
play a very important role in bone remodeling through the actions of the Wnt/β-catenin pathway regulators Dkk1 and sclerostin, both of which are highly expressed in osteocytes. Further work is required to link the ultrastructural relationship observed between osteocytes and the implant in this study with a functional role in bone formation around implants as well as the osteocytes changes caused by compromised bone environment.

The final part of our research suggested estrogen deficiency affects the jaw bone and the early healing of machined implants. Rough implants also showed faster (earlier) osseointegration compared with other groups although ultimately this difference between machined and rough surface implants was not maintained. How and why estrogen deficiency only affected the early implant healing periods and the exact mechanisms involved are still not totally understood, and deserve further research.

Finally, as this work is based on a small animal model, further work is needed using a larger animal model to confirm whether the results of this study are likely to be consistent with those in human subjects.

### 8.4 Closing Remarks

In conclusion, rat maxilla has proved to be a good model for osteoporosis research as well as a good model for dental implant research. Based on this model, we found osteocytes have a closed relationship with dental implant which indicated osteocytes may play an important role in the osseointegration remodelling process. Osteoporosis was found to reduce the early osseointegration (as measured by %BIC) of the machined implant. However, the rough surfaced implant appeared to trigger an earlier cell response able to compensate for the estrogen deficient conditions resulting in %BIC similar to that seen in SHAM animals. Nevertheless, this difference was not maintained as there was no significant difference in osseointegration between the OVX and SHAM groups in the long-term.
8.5 References

 Appendices
Chapter 4  Micro Ct data tables for Tibia and maxilla molars

Table 1  Micro CT results of tibia

<table>
<thead>
<tr>
<th></th>
<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>Conn.Dens (1/mm³)</th>
<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>Conn.Dens (1/mm³)</th>
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<tr>
<td>OXV</td>
<td>8W</td>
<td>0.036±0.060</td>
<td>0.060±0.004</td>
<td>0.980±0.368</td>
<td>6.413±1.126</td>
<td>0.148±0.038</td>
<td>0.067±0.006</td>
<td>0.284±0.054</td>
<td>3.592±0.611</td>
<td>58.787±17.926</td>
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<td>12W</td>
<td>0.031±0.010</td>
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<td>1.324±0.307</td>
<td>8.011±0.178</td>
<td>1.174±0.439</td>
<td>0.060±0.004</td>
<td>0.284±0.054</td>
<td>3.592±0.611</td>
<td>58.787±17.926</td>
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<td></td>
<td>16W</td>
<td>0.030±0.010</td>
<td>0.073±0.009</td>
<td>1.385±0.331</td>
<td>7.760±0.228</td>
<td>2.849±1.741</td>
<td>0.148±0.038</td>
<td>0.067±0.004</td>
<td>0.284±0.054</td>
<td>3.592±0.611</td>
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<td>20W</td>
<td>0.032±0.011</td>
<td>0.079±0.011</td>
<td>1.598±0.330</td>
<td>6.674±0.118</td>
<td>3.782±2.359</td>
<td>0.148±0.038</td>
<td>0.067±0.004</td>
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<td>3.592±0.611</td>
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Micro CT results of tibia showed significant differences from 8 weeks for the parameters BV/TV , Tb.Sp, Tb.Th, and Conn.dens separately. (* P< 0.05)

Table 2  Micro CT results of first molar

<table>
<thead>
<tr>
<th></th>
<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>Conn.Dens (1/mm³)</th>
<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>Conn.Dens (1/mm³)</th>
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<tr>
<td>OXV</td>
<td>8W</td>
<td>0.921±0.024</td>
<td>0.214±0.025</td>
<td>0.063±0.009</td>
<td>7.057±0.447</td>
<td>0.947±0.023</td>
<td>0.241±0.031</td>
<td>0.065±0.018</td>
<td>6.681±0.776</td>
<td>31.317±11.958</td>
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<tr>
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<td>12W</td>
<td>0.658±0.024</td>
<td>0.235±0.032</td>
<td>0.250±0.027</td>
<td>3.625±0.277</td>
<td>0.867±0.038</td>
<td>0.272±0.028</td>
<td>0.131±0.027</td>
<td>5.160±0.483</td>
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<td>16W</td>
<td>0.602±0.100</td>
<td>0.166±0.035</td>
<td>0.237±0.059</td>
<td>4.163±0.492</td>
<td>0.817±0.106</td>
<td>0.250±0.045</td>
<td>0.172±0.068</td>
<td>4.790±0.486</td>
<td>17.649±5.835</td>
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<tr>
<td></td>
<td>20W</td>
<td>0.672±0.082</td>
<td>0.209±0.024</td>
<td>0.220±0.022</td>
<td>4.296±0.357</td>
<td>0.798±0.098</td>
<td>0.257±0.034</td>
<td>0.172±0.069</td>
<td>4.363±0.467</td>
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Micro CT results of first molar showed significant differences from 12 weeks for the parameters BV/TV , Tb.Sp, Tb.Th and Tb.N . (* P< 0.05) Note the difference between the results for Tb.Th between the maxilla here and the tibia (table 1).
Table 3 Micro CT results of second molar

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<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
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<th>Tb.N (mm)</th>
<th>Conn.Dens (1/mm³)</th>
<th>BV/TV (%)</th>
<th>Tb.Th (mm)</th>
<th>Tb.Sp (mm)</th>
<th>Tb.N (mm)</th>
<th>Conn.Dens (1/mm³)</th>
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<tr>
<td>O VX</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>8W</td>
<td>0.89±0.046</td>
<td>0.22±0.014</td>
<td>0.07±0.018</td>
<td>6.97±1.06</td>
<td>47.96±16.39</td>
<td>0.84±0.051</td>
<td>0.21±0.026</td>
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<td>7.09±0.58</td>
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<td>12W</td>
<td>0.53±0.08</td>
<td>*0.21±0.026</td>
<td>*0.32±0.03</td>
<td>3.93±0.44</td>
<td>20.07±6.90</td>
<td>0.72±0.03</td>
<td>0.22±0.035</td>
<td>0.18±0.04</td>
<td>5.33±0.69</td>
<td>32.96±13.17</td>
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<tr>
<td>16W</td>
<td>0.54±0.11</td>
<td>*0.32±0.03</td>
<td>*0.22±0.05</td>
<td>4.98±0.65</td>
<td>58.07±7.95</td>
<td>0.97±0.76</td>
<td>0.25±0.049</td>
<td>0.16±0.058</td>
<td>4.68±0.57</td>
<td>28.93±9.34</td>
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<td>20W</td>
<td>0.51±0.71</td>
<td>*0.16±0.01</td>
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<td>4.20±0.50</td>
<td>51.92±8.46</td>
<td>0.81±0.10</td>
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<td>0.14±0.066</td>
<td>5.30±0.75</td>
<td>23.15±10.14</td>
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Micro CT results of second molar showed significant differences between the groups for the parameters BV/TV, Tb.Sp, and Tb.N from 12 weeks after surgery. For Tn.Th, the difference between the groups was statistically significant from 16 weeks. Note the difference of index Tb.Th compared with tibia (table 1). (*P<0.05)
### Primer Sequences Used for Real-time PCR

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<td>B2M</td>
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<tr>
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<td>R: 5’-GTCCAGATGATTGAGCTCCA-3’</td>
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<tr>
<td>GAPDH</td>
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<td></td>
<td>R: 5’-ACATACTCAAGACACCACATC-3’</td>
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<td>β-actin</td>
<td>F: 5’-CACCGCGAGTACACCTTC-3’</td>
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<td>ALP</td>
<td>F: 5’-TCGGACATGAGATGCGCC-3’</td>
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<td>R: 5’-TGGGAGTGCTTGCTAGG-3’</td>
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<td>OC</td>
<td>F: 5’-GCCCTGACTGCACTCTGCTC-3’</td>
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<td></td>
<td>R: 5’-TCACCACCTTACTGCCCTCTC-3’</td>
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<td>COL-1A</td>
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<td>R: 5’-GAATCGACTTGGCTTCGC-3’</td>
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<td>RANKL</td>
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<td>TRAP</td>
<td>F: 5’-GACCCTTTATTACCTGGGTGC-3’</td>
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<td>DMP1</td>
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<td>SOST</td>
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**Enthic approved by Fujian Medical university**

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**福建省医科大生物医学研究伦理审查意见**

<table>
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<th>研究项目名称</th>
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<tr>
<td>研究项目负责人</td>
<td>邓川 杜志斌</td>
</tr>
<tr>
<td>项目承担单位</td>
<td>福建医科大学附属口腔医院</td>
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### 研究内容概述（包括伦理有关的研究内容）

本课题采用Sprague-Dawley大鼠建立骨质疏松大鼠模型。三个月模型成熟后，拔除大鼠上颌第一磨牙同侧植入2×3mm种植体。同时使用抗骨质疏松药物

Simvastatin用于观察该药物在骨质疏松状态下种植体骨结合的影响。本课题需申请120只3月龄大鼠供研究使用。望批准。

### 审查意见

[审查意见签名]

### 伦理委员会委员会

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福建省医科大生物医学研究伦理审查委员会
Dear Prof Ivanovski

I write further to the additional information provided in relation to the conditional approval granted to your application for ethical clearance for your project "Dental implant evaluation in compromised conditions" (GU Ref No: DOH/01/11/AEC).

This is to confirm receipt of the remaining required information, assurances or amendments to this protocol.

Consequently, I reconfirm my earlier advice that you are authorised to immediately commence this research on this basis.

The standard conditions of approval attached to our previous correspondence about this protocol continue to apply.

Regards

David Rounsevell
Research Ethics Officer
Office for Research
Bray Centre, Nathan Campus
Griffith University
ph: 3735 6618
fax: 3735 7994
email: D.Rounsevell@griffith.edu.au

Cc:

At this time all researchers are reminded that the Griffith University Code for the Responsible Conduct of Research provides guidance to researchers in areas such as conflict of interest, authorship, storage of data, & the training of research students.

You can find further information, resources and a link to the University's Code by visiting http://www62.gu.edu.au/policylibrary.nsf/xupdatemonth/e7852d226231d2b44a25750c0062f457?open document

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