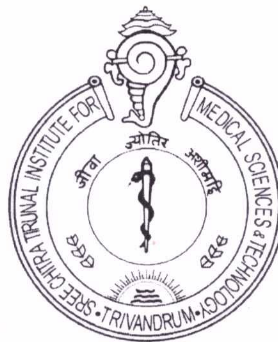
LONG TERM OSSEOINTEGRATION STUDY OF ACID ETCHED AND SLA SURFACE TREATED TITANIUM IMPLANTS IN RABBIT MODEL

A DISSERTATION SUBMITTED BY

Ms NEETHU R S

2020



SREE CHITRA TIRUNAL INSTITUTE FOR

MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM – 695011

2020

LONG TERM OSSEOINTEGRATION STUDY OF ACID ETCHED AND SLA SURFACE TREATED TITANIUM IMPLANTS IN RABBIT MODEL

A DISSERTATION SUBMITTED BY

Ms. NEETHU R S

2019/M Phil/01

TO

THE SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY THIRUVANANTHAPURAM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY IN BIOMEDICAL TECHNOLOGY

2020

DECLARATION

I, **Neethu R S**, hereby declare that I had personally carried out the work depicted in the thesis entitled, **"Long Term Osseointegration Study of Acid Etched and SLA Surface Treated Titanium Implants in Rabbit Model"** under the direct supervision of **Dr. Sabareeswaran A, Scientist F, Histopathology Laboratory**, Division of Experimental Pathology, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Thiruvananthapuram

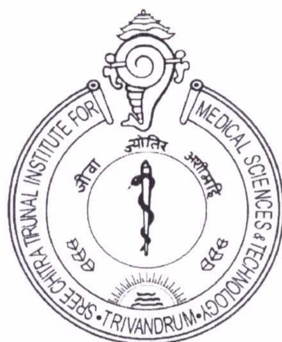
Date: 28-07-2020

Ms. Neethu R S

2019/M Phil/01

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY TRIVANDRUM – 695011, INDIA

(An Institute of National Importance under Govt. of India with the status of University by an Act of Parliament in 1980)



CERTIFICATE

This is to certify that the dissertation entitled **"Long Term Osseointegration Study of Acid Etched and SLA Surface Treated Titanium Implants in Rabbit Model "** is a bonafide work done by **Ms. Neethu R S** in partial fulfilment for the degree of **Master in Philosophy in Biomedical Technology** under my supervision and guidance at **Histopathology Laboratory**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Thiruvananthapuram

Date: 28-07-2020

Dr. Sabareeswaran A

Scientist F, Histopathology Division

The Thesis entitled

LONG TERM OSSEOINTEGRATION STUDY OF ACID ETCHED AND SLA SURFACE TREATED TITANIUM IMPLANTS IN RABBIT MODEL

Submitted by

Ms. NEETHU R S

For the degree of

MASTER OF PHILOSOPHY

IN

BIOMEDICAL TECHNOLOGY

OF

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

Thiruvananthapuram – 695011, INDIA

Evaluated and approved by

Signature: **Signature:**

Name of supervisor: A. Sabareeswaran. **Name of examiner:** Sachin J Shenoy

CONTENTS

ACKNOWLEDGEMENT……………………………………………………………….i

LIST OF ABBREVIATION..iii

LIST OF NOTATIONS..v

LIST OF TABLES..vi

LIST OF FIGURES.. .vii

SYNOPSIS...1

INTRODUCTION...3

1.1 BACKGROUND..3

1.2 REVIEW OF LITERATURE..6

1.2.1BONE...6

1.2.1.1 CLASSIFICATION OF BONE 6

1.2.1.2 STRUCTURE OF BONE..7

1.2.1.3FORMATION OF BONE...11

1.2.1.4 BONE HEALING…...13

1.2.2 IMPLANTS...18

1.2.3 TITANIUM 20

1.2.4 SURFACE MODIFICATION……..20

1.2.4.1 ACID ETCHING..23

1.2.4.2 SANDBLAST, LARGE-GRIT, AND ACID ETCHING (SLA.....................23

1.2.5 BONE SPECIAL STAINS…………………………………………….....…24

1.2.5.1 STEVENEL'S BLUE..24

1.2.5.2 VAN GIESON PICROFUSCHIN STAIN………………………….………..24

1.2.6 BONE HISTOMORPHOMETRY……………………………..…………..25

2. MATERIAL AND METHODS…..26

2.1 MATERIALS USED FOR STUDY..26

2.1.1 ANIMAL MODEL USED……………………………………………………...26

2.2 IN VIVO LONG TERM EVALUATION……………….................................26

2.3 SAMPLE COLLECTION...26

2.4 HISTOLOGICAL ANALYSIS..26

2.4.1 INSTRUMENTS USED...26

2.4.2 CHEMICALS AND REAGENTS...27

2.4.3 PROCESSING AND EMBEDDING OF BONE WITH IMPLANT.............27

2.4.4 TISSUE EMBEDDING IN PMMA………………………….………….....27

2.4.5 SECTION CUTTING..28

2.4.6 GRINDING AND POLISHING..28

2.4.7 STAINING OF RESIN EMBEDDED TISSUE SECTION.............................28

2.4.8 MIROSCOPIC EVALUATION……………………………….……………….29

2.5 HISTOMORPHOMETRY ANALYSIS………………..………………..........29

2.6 MOLECULAR STUDIES..29

2.6.1 RNA ISOLATION..29

2.6.2 cDNA SYNTHESIS..31

2.6.3 REAL TIME PCR AMPLIFICATION AND QUANTIFICATION OF
COL1 A1, SPP 1, SPARC, RUNX 2, GAPDH, VEGF, TGF-B
GENE EXPRESSION 32

2.7 RT-PCR ANALYSIS..34

2.8 STATISTICAL ANALYSIS...34

3. RESULT AND DISCUSSION...35

3.1 IN VIVO EVALUATION...35

3.2 GROSS PATHOLOGY OBSERVATION...35

3.3 HISTOPATHOLOGY OBSERVATION…………………………………….....35

3.4 HISTOMORPHOMETRIC ANALYSIS..38

3.5 MOLECULAR STUDIES..40

3.5.1 GENE EXPRESSION PATTERN………………………………...………40

4.1SUMMARY...43

4.2CONCLUSION..44

4.3 FUTURE PROSPECTS...44

BIBLIOGRAPHY...47

ACKNOWLEDGMENT

I would like to present my heartfelt thanks to Dr. Sabareeswaran A, Scientist F, Histopathology division, Biomedical Technology Wing SCTIMST, Poojappura for his valuable guidance, unceasing help, unerring supervision, critical suggestion, encouragement, stress-free environment and freedom with which I could work in his laboratory throughout the M.Phil course.

I would like to express my sincere thanks to Department of Biotechnology, Government of India, for funding the project.

I cordially articulate my sincere thanks to Dr. Asha Kishore, The Director, SCTIMST, Dr. Harikrishna Varma PR, Head, BMT Wing, Dr. Sankara Sarma P, Dean, Dr. Santhosh Kumar V, Registrar, for allowing me to do my MPhil course.

I would like to express my sincere thanks to Dr. Maya Nandakumar A, Head of the Department Applied Biology Division, Dr. Manoj Komath, M Phil Coordinators, Dr. Francis Boniface Fernandez, Dr. Anoop Thekkuveetil and Dr. Jayasree RS for their sincere help rent for the completion of my M.Phil course.

I also express my sincere thanks to Dr. Senthilkumar Muthuswamy for his timely advice, support, and patience he took to complete my work.

I express my heartfelt thanks and gratitude to lab members from histopathology laboratory Mr. Joseph Sebastian (Technical Assistant), Mrs. Sandhya Rani (Project assistant), and Mr. Viswanath Naik V (Technical Assistant) for their help and technical advice.

I would like to thank Mrs. Lekha (cleaning staff) for providing the lab clean and tidy.

I express my sincere thanks to Ms. Gayathry (Ph.D. Student, Bioceramic division) for her immense support and patience. I express my sincere thanks to the students and staff from the Division of Tissue Culture for their help to complete my work.

I express my sincere thanks to Dr. Anugya Bhatt, Mrs. Renu Ramesh and Ms. Deepa from Division of Thrombosis Research for their help and support.

I express my sincere thanks to Dr. Rekha MR, Division of Bio surface Technology, Mrs. Rajalekshmy, and Mrs. Annie for the nanodrop facility.

I express my immense thanks to Dr. Prabha D. Nair and Amrita Natarajan from Div. of Tissue Engg. & Regenerative Technology.

I express my sincere thanks to Dr. Anilkumar T. V, students, and staff from the Experimental pathology laboratory.

I express my sincere thanks to Dr. Sachin J. Shenoy and staff from the division of in-vivo models and testing for their technical support.

I owe my deep sense of gratitude to my parents for their encouragement and support to pursue my M.Phil course, above all it is the blessings of the almighty of god, which showered upon me that helped me to reach this part of my life successfully.

ABBREVIATIONS

BIC	**Bone-To-Implant Contact**
BGLAP	**Bone Gamma-Carboxyglutamic Acid-Containing Protein**
BLAST	**Basic Local Alignment Search Tool**
BMP2	**Bone Morphogenetic Protein 2**
cDNA	**Complementary DNA**
COL1A1	**Collgen, type I, alpha 1**
DEPC	**Diethyl Pyrocarbonate**
ECM	**Extracellular Matrix**
FGF	**Fibroblast Growth Factors**
GAG	**Glycosaminoglycans**
GAPDH	**Glyceraldehyde 3-**
H2SO4	**Sulfuric Acid**
HCl	**Hydrochloric acid**
HF	**Hydrofluoric acid**
IEC	**Institutional Animal Ethics**
IGF-I	**Insulin-like growth factor 1**
IGF-II	**Insulin-like growth factor 1**
Ihh	**Indian hedgehog**
IL-1	**Interleukin-1**
IL-6	**Interleukin-6**
ISO	**International Organization for Standardization**
$MgCl_2$	**Magnesium chloride**
MMA	**Methyl Methacrylate**
MSC	**Marrow Stromal Cells**

NBF	**Neutral Buffered Formalin**
OB	**Osteoblasts**
OCN	**Osteocalcin**
OD	**Optical Density**
ON	**Osteonectin**
OPG	**Osteoprotegrin**
OPN	**Osteopontin**
PCR	**Polymerase Chain Reaction**
PDGF	**Platelet derived growth factor**
PLA	**Platelet-lymphocyte ratio**
PMMA	**Poly(methyl methacrylate)**
PTHrP	**Parathyroid hormone-related protein**
RNA	**Ribonucleic acid**
RT PCR	**Reverse transcription polymerase chain reaction**
RTase	**Reverse transcriptase**
RUNX 2	**Runt-related transcription factor 2**
SLA	**Sandblasted acid etched**
SPARC	**Secreted protein acidic and rich in cysteine**
SPP1	**Secreted phosphoprotein 1**
TGF β	**Transforming growth factor beta**
Ti	**Titanium**
TiO2	**Titanium dioxide**
TNF α	**Tumour Necrosis Factor alpha**
VEGF	**Vascular endothelial growth factor**

LIST OF NOTATIONS

h	Hour
min	Minute
sec	Seconds
cm	Centimeter
cm^2	Centimeter square
M	Molar
Mg	Milligram
g	Gram
µg	Micogram
µl	Micoliter
µm	Micometre
ml	Milliliter
°C	Degree Celsius
%	Percentage

LIST OF TABLES

Table No	Caption	Page No
1	Cells present in bone and their characteristics	10
2	Proteins invoved in osseogenesis	11
3	Mastermix protocol for cDNA synthesis for 10µl	32
4	list of primers for gene expression study	33
5	RT-PCR master mix protocol	33

LIST OF FIGURES

Figure No	Caption	Page No
1	Structure of bone	7
2	Fracture Healing	15
3	Signalling cascade-Fracture healing	17
4	Surface modification	22
5	Histomorphometry analysis-Bone to implant contact	24
6	Femur cross section- Stevenel’s Blue stain Van Gieson Picrofuschin stained microscopic images of the bone with implant interface	36
7	Femur cross section- Stevenel’s Blue stain Van Gieson Picrofuschin stained microscopic images of the bone with implant interface	37
9	Graphical representation of Percentage of Bone to implant contact in Femur	38
10	Graphical representation of RT-PCR data-COL1A1,RUNX2,SPARC and SPP1	39
11	Graphical representation of RT-PCR data-TGF β	41
12	Graphical representation of RT-PCR data- VEGF	42

SYNOPSIS

SYNOPSIS

Titanium (Ti) implants are the most preferred choice for orthopedic implants as it has good biocompatibility, mechanical stability, and chemical stability will promote osseointegration when compared to other metal implants. Surface modification has direct influence on the osseointegration properties, the surface modifications on the Ti implant increase the area of the implant which will improve osseointegration property of the implant. In this study, a systematic approach has been employed to study the osseointegration ability of SLA treated and acid etched Ti dental screw implants.

Chapter 1: This chapter begins with introducing the background of the study, literature review and hypothesis of this study Literature review focuses on the bone tissues, natural bone healing mechanism includes molecular mechanism. Ti implants and its surface modifications were included in this section. Hypothesis that we have postulated based on the literature survey, aim of the study along with the objectives of this study has been described at the end of this chapter.

Chapter 2: The materials used in this study and the method followed were detailed in this chapter. Gross histopathological analysis and histomorphometry was performed in this study for analysing the qualitative and quantitave analysis of new bone formation respectively. Histology includes the PMMA resin embedding, sectioning and staining of bone with implant. Standard protocol was followed for this Stevenel's blue stain and Van Gieson stain is used for the histological analysis. From the histology photomicrograph images histomorphometry analysis was done using a standardized formula. Molecular studies have been performed to analyse the quantitative gene expression of selected genes involved in osteogenesis. The primers for the gene expression study were designed using BLAST. GAPDH gene was selected as the reference gene and the expression of selected osteogenic, angiogenic and inflammatory markers were evaluated in test and control group. Protocol provided by the kits was used for RNA isolation and cDNA synthesis. Quantitave gene expression analysis was done by using qRT-PCR technique.

Chapter 3: Results and discussion obtained from the study is detailed with supporting data in this chapter. Gross and histopathology analysis revealed the absence of inflammation, necrosis and degeneration in both groups. New bone formation and osseointegration were observed in both groups. Photomicrographs of Stevenel's blue and Van Giesons picrofuschin stained sections obtained from PMMA resin embedded tissue sample were included. Histomorphometric analysis and data from the captured microscopic image were presented. The relative gene expression of selected osseogenic genes such as COL1A1, RUNX2, SPARC, SPP1, angiogenic gene VEGF and inflammatory marker gene TGF β has been given in detail in this chapter. A graph representing the fold change of genes expressed in test and control group has been included. Statistical analysis of result is also provided in this chapter. This chapter also discusses the results of surface modified implants obtained from this study.

Chapter 4: The final chapter summarizes the findings from this study. This chapter concludes the osseointegration ability of the surface modified implants based on the results obtained in this study. The future perspective of this study is also provides at the end of this chapter.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1. BACKGROUND

Bone is the structural base for the skeletal system; apart from mechanical functions such as providing structural support and strength, it protects the internal organs from injuries and function in haematopoiesis. They will provide mobility and strength by storing minerals especially calcium. The lamellar and spongy pattern of bone tissue gives rigid structure to the bone. Bone cells are classified into three, osteoblast, osteocytes and osteoclasts cells accordance with the role in bone formation.

Osteoblasts are cells with single nucleus; during bone formation these cells function as group to synthesize bone. The bone mineral hydroxy appetite was produced by these osteoblast cells and the hydroxy appetite deposition will gives a strong and dense mineralized tissue matrix, which supports body. The minerals stored in the matrix helps to maintain homeostasis, acid-base balance and essential chemical balance such as calcium and phosphate.

Osteoclast is a type of bone cell that function in breaking down the bone tissue. Bone breaking helps to maintain the structure and remodelling of bone by repairing. Bone resorption is process in which the osteoclasts cells disassemble and will digest the minerals and proteins by secreting collagenase.

Osteocytes are the most abundant cell type present in the matured bone. They reside inside the small chamber called lacuna, which are enriched with calcified bone matrix. Osteocytes functions to maintain the bone integrity by regulating bone formation through signalling pathways. They derived from the osteoblast cells.

Osseogenesis is the process of bone formation; it can be either by endochondral ossification or by the intramembranous ossification. Endochondral ossification is the process in which bone will form from the cartilage (normally the bone formation is done by this method).There are natural mechanism to heal the bone wound and breaks by the body itself, but some conditions like deep wounds and serious bone

fracture the mechanism itself cannot cure the problem. To overcome these problems implants and guided bone regeneration mechanism can be done.

Osseointegration is the direct structural connection between the bone and implant. This is the important feature of implant to be considered as good implant for long term implantation. The osseointegration will enhance the bone resorption and remodelling. There are many proteins involved in this process in order to maintain the integrity of bone. Osteopontin (OPN), Osteonectin (ON), Osteocalcin (OCN) and Osteoprotegrin (OPG) are the important proteins involved in osteointegration.

Angiogenesis is the normal physiological process through which the new blood vessels formed from the pre-existing blood vessels; VEGF plays a crucial role in angiogenesis. VEGF promote to restore the blood supply to cells and tissue. They act as signalling protein for the new blood vessel formation.

TGF-β is a multifunctional cytokine, which stimulate osteoblast cell replication and promotes bone formation. TGF-β functions in the regulation of cell proliferation, cell differentiation and extracellular matrix production.

Presently, the implants are so well developed when compared to the earlier time periods. Earlier, the implant materials used were not that much effective for long term implantation which resulted in implant failure and patients had to undergo further surgeries. To make the implant better and reducing the number of surgeries, implant modifications were introduced. Implant modification can be done by different ways .The surface modified implants will give better results than the surface unmodified implants. The Titanium implants were used as orthopaedic implant for decades. Now the optimal metal choice for orthopedic implant was Ti. It used as head to toe implantation process. Ti has improved biocompatibility than other metal; in dental and orthopaedic implantation .Surface treated Ti implants were used now in order to achieve the long term implantation without much failure. From many studies it has been observed that the surface treatment has shown a remarkable improvement in the osseointegration property.

In this study, surface modified Ti dental implants have been used to understand the new bone formation and osseointegration. For the evaluation a systematic approach was undertaken. The surface modified Ti implants were implanted on the femoral condyle of healthy New Zealand White rabbits for period of six months. The bone tissue samples with implant were evaluated to understand the osseointegration ability of surface modification done on Ti implants using molecular studies and histological analysis.

1.2 REVIEW OF LITERATURE:

1.2.1 *BONE*

Bone is the hardest organ, which offers a rigid structure to the body and facilitates mobility and helps to protect internal organs. They are dynamic in nature and act as a mineral reservoir for calcium and phosphorous. The organic portion of bone is consist of cells and is embedded within the bone matrix. Collagen is the most abundant structural protein present inside the bone matrix. The inorganic part of bone is majorly consists of calcium in the form of hydroxyapatite. Bone is actively built and remodelled in the course of life by unique bone cells called osteoblasts and osteoclasts. In body bones exist in different shapes.

1.2.1.1 *Classification of bone:*

- Long bone
- Short bone
- Flat bone
- Irregular bone

Long bones:

These types of bones are usually seen in the arms and legs (humerus, ulna, femur, and tibia). They are cylindrical in shape. They have spongy bones at the ends and the long bone provides an attachment site for muscles. Long bones have four remarkable regions: *the epiphysis, metaphysis, physis, and diaphysis*. The *epiphysis* is typically a secondary centre of ossification placed on the cartilaginous ends of long bone (articular surface) and capabilities to give out stress throughout the joint. The *metaphysis* is a transition zone among diaphysis and epiphysis, and is composed of common trabecular bone. The *physis* or growth plate is accountable for longitudinal bone boom by means of endochondral ossification until skeletal development occurs. The physis is interspersed between the epiphysis and the metaphysis. It is absent in adults. The diaphysis consists of the central portion of the bone, and it could be taken

into consideration as a tubular cavity containing the foremost arterial supply to the endosteum(Stewart et al., 2015).

Short bones:

They have a cube like shape; they are equal size in width, length and thickness. These bones are present in the wrists and torsals of ankles. Their function is to provide stability and support with limited mobility.

Flat bones:

They are present in cranium of skull, scapulae in shoulder blades, sternum (breastbone), and the ribs. This bone provides an attachment site for muscles and they protect internal organs.

Irregular bones:

They does not have any characterized shape, e.g.; sinus containing facial bone they are complex in shape so they can not classified on the basis of shape .They are functioning to protect spinal cord from stress.

1.2.1.2 Structure of bone:

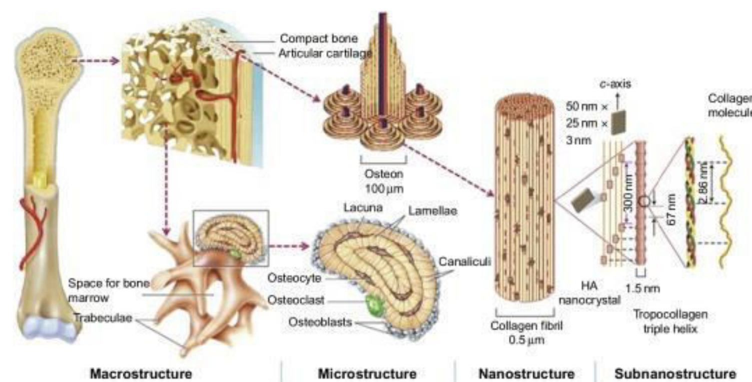


Figure: 1 Structure of bone

Meskinfam M. Polymer scaffolds for bone regeneration *Characterization of Polymeric Biomaterials*

Bone has a complex structure, each structural unit have a characterized function. The main function of bone is to provide strength, support and mechanical structure to

muscles and soft tissues. Bone has a complex structure which includes mineral phase and organic phase. The mineral phase usually consists of bio mineral, hydroxyapatite. The organic phase is rich in cells, proteins, and few lipids(Fatty acids, cholesterol, phospholipids and several endogenous metabolites) which has a major role in bone healing and bone mineralization(During et al., 2015).

Bone can be classified into two; in accordance with the macro structural level

- Trabecular bone, which is also called spongy or cancellous bone (20% of total skeleton)
- Cortical bone, which is also called compact (80% of total skeleton)

These bones are differing in functions and density. The structure and mechanical property of cortical bone helps to provides resistance to stress and tension while spongy bone mechanical structure gives resistance to compression(less extent while comparing with cortical bone). Cortical bone has compact structure, less porous, having lower blood vessel concentration compared with spongy bone. Cortical bone gives the mechanical structure to the flat bone, irregular bones, short bones and long bones.

Spongy bone or trabecular bone is highly porous and has high blood vessel concentration. They are usually present in the metaphysis of long bones. Cortical bone gives a protective covering to trabecular bone. The trabecular bone's single unit is called trabeculae, a microscopic tissue element present the trabecular bone seen as small rods or bars which supports the framework of bone(Meskinfam, 2017)

Mineral phase:

The major component of bone mineral part is hydroxyapatite. They give rigidity to bone and teeth. Hydroxyapatite is occurring in nature as calcium apatite mineral form $(Ca_5 (PO_4)_3(OH)$. They are seen as crystallized form in bone. Hydroxyapatite in its carbonated calcium-deficient form is the main mineral component in dental enamel and dentin. Hydroxyapatites having biological or synthetic origin are widely used in bone repair and bone regeneration. They are commonly used as scaffolds, blocks and

granular form. They can use directly or composite them with ceramics and polymer as coating in the orthopedics (Legeros and Legeros, 2008).

In addition to hydroxyapatite, other minerals are also present in small quantity in the bone mineral phase such as, octa-calcium phosphate (OCP),amorphous calcium phosphate (ACP) ,which are act as the precursors for hydroxyapatite (Qiu et al., 2019).

Organic phase in bone:

The cells present in the bone include osteocytes, osteoblasts, osteoclasts and mesenchymal progenitor cells.

Osteoblasts: These type of single nucleated cells forms new cells; they are originated from bone marrow. The newly formed bone is called osteoid, which is rich in proteins. Osteoblast has major role in mineral deposition in bone, they produce hydroxyapatite in highly regulated manner in organic matrix thus will leads to the mineralized matrix formation. Osteoblast serves the major cellular phase in bone; they arise from mesenchymal stem cells. Periosteum of bone is enriched with osteoblast cells while comparing with endosteum.

Osteocytes: Osteocytes are present in the mature bone; they are derived from osteoprogenitor cells. They are differentiated from osteoblast cells. Mature osteocytes are surrounded by mineralized bone. They exhibit a star shaped cell with slender cytoplasm processes, which radiated into all direction and perpendicular to the bone surface. The functions of osteocyte cells is in bone formation and bone remodelling phase (Bonewald, 2010).

Osteoclasts: These cells have hematopoietic origin and they have a major role in bone repair, remodelling and regulating blood level calcium. They reside in a small cavity known as Howship's lacunae. They are capable to digest the hydrated proteins in bone by collagenase this process is called bone resorption. During the resorption phase the calcium level is regulated. The osteoclasts cells have a homogenous foamy

cytoplasm due to the higher concentrations of vacuoles including lysosomes (Parvizi and Kim, 2010).

Cell Type	Morphology	Location	Function
Osteoblast	Cubic structure with protrusions	Seen as covering layer on the surface of new bone	promote osteoid secretion and calcification
Osteocytes	Small cells with protrusions having flat oval shape	Scattered in the bone plate or between the plates	osteogenic and osteolytic activation, regulate the level of calcium and phosphorus
Osteoclasts	Multinucleated giant cells	In the tissue edge	Bone resorption

Table: 1 Cells present in bone and their characteristics

Qiu, Z.-Y., Cui, Y., Wang, X.-M., 2019. Natural Bone Tissue and Its Biomimetic, in: Mineralized Collagen Bone Graft Substitutes. Elsevier, pp. 1–22.

Proteins: The organic phase of bone contains collagenous and noncollagenous proteins. Collagen is most abundant structural protein present in extra cellular matrix of bone. Non collagenous proteins present are osteocalcin osteonectin osteopontin, bone sialoprotein, proteoglycans, glycoproteins, decorin, phosphoproteins, thrombospondin, fibronectin and phospholipids.

Proteins	Function
Collagen 1	bone structure and matrix mineralization(Type I,II,III,IV etc)
Osteonectin (ON)	Bind with calcium secreted by osteoblasts initiate mineralization and promote mineral crystal formation (SPARC)
Osteocalcin (OC)	secreted by osteoblasts remodeling of the bone, metabolic regulation (BGLAP)
Osteopontin (OPN)	bone remodeling and regulate bone mineralization(SPP1)

Table: 2 Proteins involved in osseogenesis

Qiu, Z.-Y., Cui, Y., Wang, X.-M., 2019. Natural Bone Tissue and Its Biomimetic, in: Mineralized Collagen Bone Graft Substitutes. Elsevier, pp. 1–22.

1.2.1.3 Formation of bone

Formation of bone is called osseogenesis (ossification). The two types of ossification processes are;

- Intramembranous ossification
- Endochondral ossification.

Intramembranous ossification*:* is the direct differentiation of progenitor cells to osteoblasts, which produce bone. Intramembranous ossification is the primary mechanism by which bone forms in embryonic stage. The intramembranous bone formation also occurs during bone regeneration, and especially is that the principal means of bone formation in primary bone healing, during which no secondary cartilaginous intermediate is employed. This primary bone formation can occur when a bone is much stabilized. During the method of intramembranous bone formation a set of undifferentiated osteoprogenitor is invaded by endothelial cells to supply initial blood supply, and through cell signalling pathways, the cells differentiate into osteoblasts that deposit organic bone matrix that mineralizes to make bone.

Endochondral ossification*:* Other axial and skeletal bones are formed by the method *endochondral ossification*. Endochondral bone formation occurs in embryonic bone growth, longitudinal bone growth at the physis, and within the majority of fracture calluses. During the initial formation of a bone, mesenchymal condensates differentiate to make cartilaginous anlagen that are then invaded by blood vessels. The blood vessels functioning as a route of transit for osteoblasts that then form bone on the cartilagenous template that's partially destroyed by the activity of clastic cells. During growth, endochondral ossification occurs at growth plates (physis). Chondrocytes align themselves in columns parallel to the long axis of the bone and differentiate into histological distinct zones. The resting cartilage zone is found on the brink of the epiphysis and consists of metabolically inactive chondrocytes(Stewart et al., 2015). Bone regeneration utilizes endochondral ossification to rapidly form a soft callus which will then undergo ossification. Bone features a high rate of metabolic activity with constant resorption and formation through osteoblast and osteoclasts activity(Stewart et al., 2015).

Vascular nature of bone leads to a rapid response to physical and biochemical forces and a rapid rate of repair. Woven bone is that the first to seem during regeneration and consists of an osteoid matrix with osteoblasts and collagen fibres. As healing progresses, woven bone remodels and is gradually replaced by lamellar bone. Lamellar bone consists of collagen fibres, Haversian systems, and interstitial lamellae. The functional unit of bone is that the osteon or Haversian system. At the middle of every osteon there may be a channel referred to as a Haversian canal, containing blood, lymphatics, and nerves vessels. Osteocytes are located within the small spaces on the periphery of every lamella called lacunae. The periphery of every osteon may be a narrow region of a cement-like substance composed of GAGs.

There are two distinct sorts of osteon in lamellar bone: primary and secondary. Primary osteons are liable for appositional bone growth and are related to the Haversian canal system. They run parallel to the long axis of the bone and are always surrounded by woven bone. Secondary osteons are continuous with the continued remodelling process and are initiated by osteoclastic resorption of bone. They

contain concentric sheets of lamellar bone and are bounded by cement lines.(Stewart et al., 2015)

Bone is continuously remodelled through a finely balanced equilibrium of cell-mediated extracellular matrix (ECM) degradation and formation. Bone remodeling is a versatile and ubiquitous process providing the mechanism for adaptation to mechanical stress, repair of micro-damage, and replacement of primary bone during osseointegration of implanted materials. Because it can occur in separate areas asynchronously, locally generated regulatory factors and environmental cues ensure the appropriate balance among OCs, OBs, and their precursors. Although it seems counter intuitive, implant materials for dental and orthopedic applications should not inhibit OC-mediated bone resorption to promote net new bone formation, but instead, promote a healthy communication among these cells to achieve osseointegration and maintain its longevity. (Lotz et al., 2018)

1.2.1.4 Bone healing

The method of fracture healing depends largely on inter fragmentary motion or strain within the fracture gap. Small bone defects and fractures with a well-reduced stable fixation heal by direct bone deposits and minimal callus formation with intramembranous bone formation and haversian remodelling across the fracture gap. This is often mentioned as primary bone healing. The bulk of fractures heal by callus formation and endochondral ossification or secondary healing. The response of bone to injury begins with an inflammatory phase. Bleeding from the fracture and surrounding soft tissue leads to the formation of a fibrin clot and fracture hematoma. Subsequently, there's an inflammatory cytokine release with angiogenesis, then mesenchymal progenitor cell proliferation. These mesenchymal progenitor cells are primarily derived from the periosteum and therefore the endosteum, although some studies suggest that cells can also be contributed from the muscle, the marrow, or circulation (Knight and Hankenson, 2013).

The mesenchymal progenitors rapidly proliferate, forming the initial soft callus. Soft or primary callus response occurs within a fortnight of injury. The degree of callus

formation is proportional to the degree of motion at the fracture gap. Within the second stage of repair, there's resorption of the necrotic bone ends, and therefore the mesenchymal progenitor cells that have proliferated at the location of injury begin to undergo differentiation to chondrocytes to make a cartilaginous callus, also on osteoblasts for intramembranous bone formation at the fracture margins. The mechanisms that control influx, proliferation, and therefore the differentiation capacity of the mesenchymal progenitor cells are key components of the fracture healing process (Stewart et al., 2015).

Bone healing occurs in two modes depends on bone lesions, the morphology and structure of the tissue, and fixation method.

Primary healing: osteoblasts secrete an osteoid matrix for future mineralization (intramembranous ossification)

Secondary healing: chondrocytes produces cartilage matrix later will replaced by osteoid matrix with subsequent mineralization (Lindahl et al., 2015)

The commonest growth factors associated with the bone healing, osteoinduction and osteoconduction are: PDGF, BMPs IGFs TGF-β, FGF and VEGF Local vascularisation at the location of injury has been identified together of the foremost important parameters that influence the healing process (Khojasteh et al., 2013)

Bone formation can only proceed successfully if the tissue is satisfactorily vascularised therefore, angiogenesis may be a key component in bone repair. New blood vessels will carry oxygen and nutrients for the metabolically active callus thus allowing gas exchange, and therefore, the output of waste products and function a route for inflammatory cells, and cartilage and bone precursor cells and also provide the gateway of systemically circulating factors which will modify the bone healing process. Vascularisation is required for both the formation of intramembranous and endochondral bone. Cartilage taken up blood vessels and allow osteoblastic, chondroblastic, and progenitor cells to deposit new bone on the surface. In

intramembranous ossification, vascularisation is additionally need to permit osteoblast precursor cells (Hankenson et al., 2011)

Angiogenesis and migration of vascular endothelial cells are stimulated by pro-angiogenic factors like VEGF, BMPs, TGF-β, FGF, and angiopoietins. The third stage, the transforming phase of bone healing occurs, whose main objective is to reshape the bone to revive its original structure and strength. During this phase, osteoclasts reabsorb recently formed bone tissue, thanks to the stimulation of growth factors and cytokines that promote osteoclastogenesis as TNFα, TGF, and BMPs.

Regenerated bone has good mineralized matrix and has shown increase in density by the action of osteoblast, osteoblast cells will deposit more osteoid and phosphate. Due to this action the transverse diameter of the bone decreases .Then cellularity is gradually reduced and bone density will enhance (Chaparro and Linero, 2016).

Stages of bone healing:

In fracture healing original anatomic structure and mechanical function of bone is restored, by dynamic interplay of biological processes. This involves sequential stages,

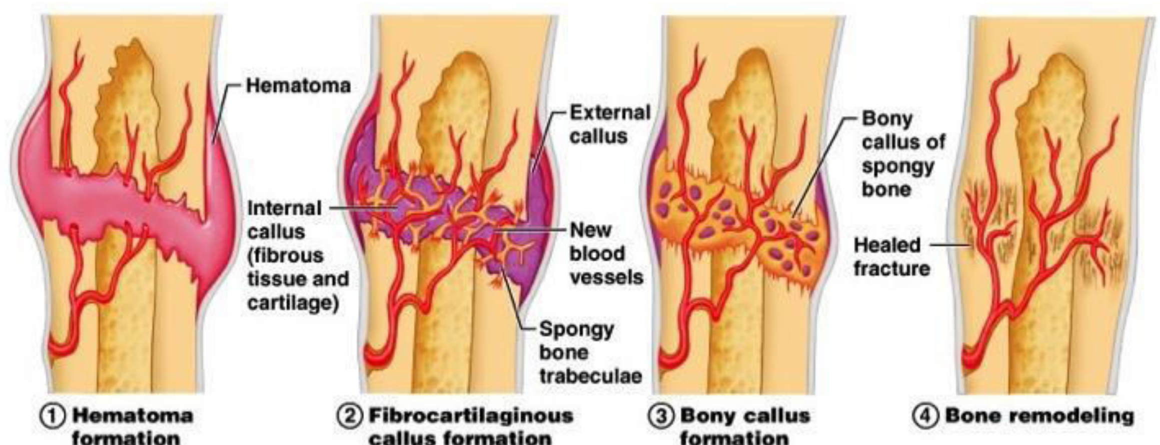


Figure: 2 Fracture Healing

Fracture Healing - Basic Science - Orthobullets https://www.orthobullets.com/basic-science/9009/fracture-healing .

- **Hematoma formation:**

In the first stage of bone healing, the blood vessels will break which leads to hemmorrhage. The hematoma formed will contains both peripheral and intramedullary cells. It provides the hematopoietic cells which are responsible for the growth factors and inflammatory cells. The inflammatory response will aid the progression in bone healing process. In this stage release of inflammatory cytokines takes place, such as TNF-α, TGF-β, IL-1, and 6, 10. Mesenchymal cells attaches to the fracture site and granulation takes place.

- **Bone generation:**

Primary callus formation occurs in two weeks after injury. Bridging soft callus formation takes place in order to connect the bone ends. The inflammatory cytokines promotes the new blood vessel formation. Angiogenic marker VEGF will release during this stage and osteoblasts and osteoclasts differentiation take place. The osteoblasts differentiation occurs, endothelial cells, fibroblasts participate in filling the fracture gap. This process results in the granulation. In normal injury the inflammatory stage is fast. In this stage the fibrous cartilage formed will convert to bony callus of spongy bone.

- **Bone remodeling:**

In the final stage of bone healing bony callus is remodelled by osteoclasts and osteoblasts. To create bone tissue that is similar to the original compact bone is added. This can take many months(Fracture Healing - Basic Science - Orthobullets,2018)

Signalling cascade on bone remodeling after fracture

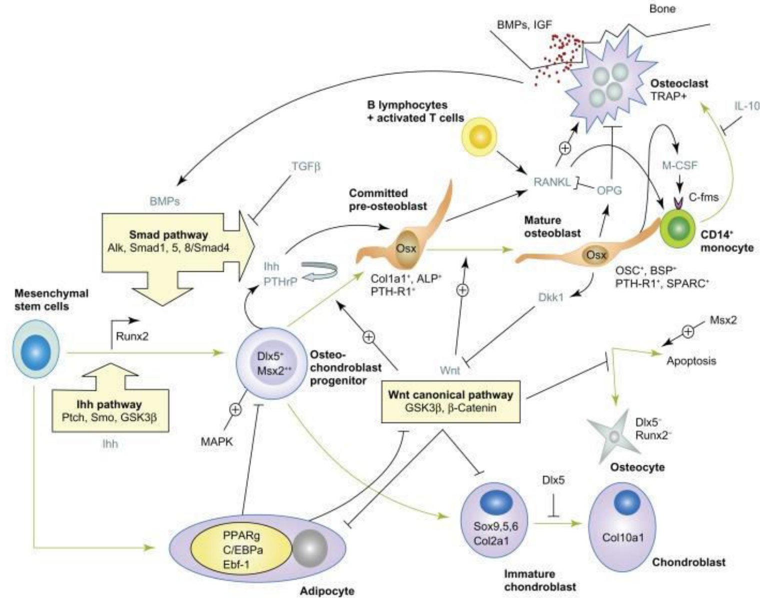


Figure: 3 signalling cascade-Fracture healing   Neman, J., Hambrecht, A., Cadry, C., Jandial, R., 2012. Stem cell-mediated osteogenesis: therapeutic potential for bone tissue engineering. Biologics 6, 47–57.

Bone remodelling involves a complex of proteins; the major osteogenic markers are COL1A1, RUNX2, and SPARC. These facilitate bone remodelling and mineralization.

Molecular markers:

COL1A1: supports and strengthens tissues function includes rigidity and elasticity. They arrange themselves into thin, long, fibrils which will cross-link to one another result in the formation of very strong mature type I collagen fibers.

SPP1: SPP1 is an osteopontin marker; they are the non collagenous protein present in the bone matrix. They have an important role in bone mineralization and

remodelling. They exist as phosphorylated form in the bone matrix. They produce osteoblastic and mesenchymal stem cells (Noda and Denhardt, 2008).

RUNX2: They have a major role in osteoblast and chondrocytes differentiation (Lam and Zhang, 2015). They are member of the RUNX family of transcription factors and having a Runt DNA-binding domain. They act as a scaffold for nucleic acids and regulatory factors which involved in skeletal gene expression. They are identified in preosteoblasts. First transcription factor required for osteoblast determination and commitment are RUNX2.

SPARC: SPARC protein is an acidic extracellular matrix glycoprotein that plays a vital role in cell-matrix interactions, bone mineralization, and collagen binding. They also increase the matrix metallo proteinases production and activity. They have functions, regulating the mineralization of bone and cartilage. They can bind collagen and hydroxyapatite (Foster et al., 2018).

VEGF: They are heparin-binding, endothelial-specific, angiogenic growth factors. They are having crucial role to the normal development and maintenance of the vascular and lymphatic systems they will increases vascular permeability. A mature bone should be vascularised(Maniscalco and D'Angio, 2006).

TGF-β: They are multi potent growth factors having capacity in cell proliferation, differentiation, apoptosis and matrix production. They promote osteoblastic cell replication and ossification .They having a biphasic effect on osteoclastogenesis (Abou-Ezzi et al., 2019).

1.2.2 Implants:

"A medical device manufactured to replace a missing biological structure, support a damaged biological structure, or enhance an existing biological structure"

- Wong et al., 2012

The implants are intended to treat or replace a missing structure, they should have good biocompatibility, bio mechanical property and they should promote

osseointegration, in case of orthopedic implants. To treat bone healing many types of implants have been used (pins, rods, screws, and plates). The implants can be made from polymer, ceramics and metals with respect to their function. The implant surface has been recognized to be a critical factor for the achievement of osseointegration. The foremost important surface properties are topography, chemistry, surface charge, and wet ability. Surface properties affect processes like protein adsorption, cell-surface interaction, and cell/tissue development at the interface between the body and therefore the biomaterial, all of which are relevant to the functionality of the device (Buser et al., 2004). In case of the orthopedic implants the most expected property from an implant is it should have better osseointegration property;

"Osseointegration (from Latin osseous "bony" and integrate "to make whole") is the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant"

-Albrektsson et al. in 1981

Bioactivity is the main requirement for a biomaterial to function properly in a bony site. It includes good biocompatibility favouring bone-like apatite formation, appropriate mechanical properties (Yaszemski et al., 1996).

Bioactivity is often induced on the surfaces of non-bioactive materials either by forming thin ceramic phases like oxides that have the power to make the functional groups or by the formation of functional groups directly that are ready to induce bone-like apatite formation on exposure to a liquid body substance.(Kokubo et al., 2003). Bioactive materials have the power to make an apatite layer on their surfaces within the body and bond to living bone through this bone-like apatite layer. Bone-like apatite is hydroxyapatite with a coffee crystallinity index. The formation of bone-like apatite layer coating on the surface of substrates is predicted to be a useful technique to induce bioactive substrates, which may be done by immersion of the substrates during a solution simulating the liquid body substance when some functional groups are formed on the substrate surfaces (Abdelrahim et al., 2016).

1.2.3 Titanium

Titanium (Ti) and its alloys are generally regarded to possess good biocompatibility. They typically don't suffer from significant corrosion in any of the biological environment. Ti readily adsorbs proteins from biological fluids. Some specific proteins like albumin, laminin V, glycosaminoglycans, collagenase, fibronectin, complement proteins, and fibrinogen are found to be adsorbed onto the surface of Ti. Ti surfaces also can support cell growth and differentiation. After the implantation it's generally accepted that osteoprogenitor cells migrate to the implant site and differentiate into osteoblasts. The primary stage within the reaction after the materials which are implanted into the body is nonspecific protein adsorption.(Zhu et al., 2004)

Titanium implants gives comfort, with no significant clinical ill effects. Ti and its alloys are generally regarded to possess good biocompatibility mechanical resistance, and corrosion resistance, but due to the risk of postoperative infections, protracted healing time (4–6 months). In the presence of thin surface oxide of TiO2, it has good corrosion resistance. They typically do not suffer from significant corrosion in any of the biological environment. Ti readily adsorbs proteins from biological fluids (Yadav et al., 2017).

Titanium (Ti) and its alloys are choice materials for both dental and orthopedic implants due to their ability to make a passive oxide layer bestowing high biocompatibility and corrosion resistance. Due to its stability in biological systems, Ti has been used extensively to investigate the process of osseous wound healing and osseointegration. When grown on Ti surfaces that are micro structured and hydrophilic, mesenchymal stem cells (MSCs) and osteoblasts (OBs) produce factors that make an osteogenic environment by stimulating osteoblastic differentiation in progenitor cells distal to the implant; promoting vasculogenesis; reducing inflammation; and regulating osteoclastic resorption to realize net new bone formation (Lotz et al., 2018).

Nanostructure titanium may have better osseointegration than titanium with micro-scale surface modifications because nanoscale surfaces have a bigger area and should better mimic the extracellular matrix to facilitate rapid bone accrual and, hence, promote the adsorption of proteins, cell adhesion and proliferation, gene regulation, and tissue integration (Zeng et al., 2019).

1.2.4 *Surface modification:*

The standard of dental implants depends on the properties of the surface to possess good interaction of the tissue and osseointegration, materials' biocompatibility and roughness of the surface played a crucial role. The increased roughness can simultaneously increase the area of the implant, improve cell migration and attachment to the implant, and enhance the osseointegration process. Past literatures have revealed most of the surface treatments ready to bring an honest effect to dental implants. The coating is proved to extend the area of the implants substantially (Jemat et al., 2015).

To serve for an extended period of time without rejection and to unravel the medical problems, Ti implant surfaces are coated with specifically engineered bioactive materials and various elements could also be implanted on the surface of biomaterial to enhance its bioactivity and stimulate bone formation for better osseointegration.(Abdelrahim et al., 2016). Osteointegration and bone regeneration requisites improved bone-targeting implant-bone interface mainly as osteoporotic materials. Regarding this needs many strategies have been proposed by various studies such as surface topography manipulation, using biomimetic coatings, and delivering drugs that enhance bone formation and inhibit bone resorption (Liu et al., 2016). The surface properties like, chemical composition, surface energy, roughness, and topography, will have a direct effect on the osteoblast differentiation. Surface roughness modification will modulate bone apposition. The surface roughness can be macro-, micro-, and nanometer-sized in texture. Macro- and micrometer surface roughness will promote the implants mechanical anchorage to bone. Nanometre roughness will aid the adhesion of osteoblastic cells and

adsorption of proteins (Ting et al., 2017). Tailored surface microstructures of 3D-printed implants might be regulated in place by changing printing parameters. The tailored surface microstructures played an important role within the biocompatibility of the implants (AO et al., 2017).

Surface modification of Ti implants can drop off the harmful effect of the implant failures and promote the osseointegration property. Physical modifications like sand blasting, plasma treatment, vapour deposition, laser processing. The electric modification includes anodizing, micro arc oxidation, and sol-gel methods. Chemical treatments are alkali heat treatment, acid base two step process treatment. Biological activity modification includes self assembly technology, bio molecule adsorption and antibacterial coating(Sivaraman et al., 2018). (Yang et al., 2019).

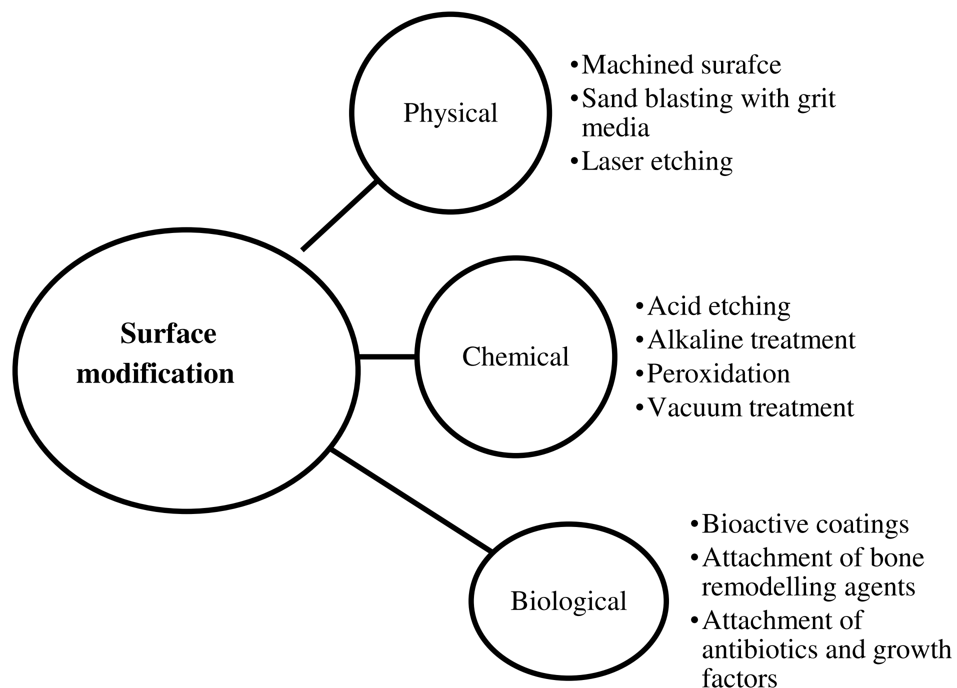


Figure: 4 Surface modifications

Ting, M., Jefferies, S.R., Xia, W., Engqvist, H., Suzuki, J.B., 2017. Classification and Effects of Implant Surface Modification on the Bone: Human Cell–Based In Vitro Studies. Journal of Oral Implantology 43 1 , 58–83. doi:10.1563/aaid-joi-D-16-00079

1.2.4.1 Acid etching:

The acid-etched implant is produced by treating with strong acid (HCl/H_2SO_4 mixture or 2% HF/10% HNO_3). This method will help to remove the grains and grain boundaries. This selective removal will provides surface roughening. The surface roughening is sensitive to the microstructure of the surface, implant material, soaking duration and the type of acid. Commonly used strong acid are nitric (HNO_3), hydrofluoric (HF), and sulphuric (H_2SO_4) or a combination of these acids. The rate of etching has a direction connection with the type and concentration of acid used for the treatment study has reported that the rate of etching depends on the type and concentration of the acid used(Ban et al., 2006). Studies have shown that the acid etched implant surfaces promoted the osseointegration by increase in cell adhesion and bone formation. This surface modification will aid the homologous roughening to the implant surface which will promote the cell adhesion. The acid etched Ti implants has shown that the surface treated with acid etching technique has improved their stability of bone formation. To get further improvement in surface modification by acid etching, the implants needs to be treated with particles before subjecting to acid etching. The etching treatment will remove the blasted particles and will provide surface roughening(Jemat et al., 2015).

1.2.4.2 Sandblast, Large-Grit, and Acid Etching (SLA):

Sandblasting and acid etching (SLA) treatment is one of the foremost widely used surface treatment methods for titanium implants. The surface properties, like hydrophilicity and functional OH groups, play a crucial role in bone fixation in vivo. Plasma treatments and acid etching (SLA) methods will produce functional OH groups on the rough surface, is usually used as a surface treatment because it produces a high contact ratio with micron and submicron roughness surface. SLA is usually utilized in commercial implant surface treatment. (Starosvetsky and Gotman, 2001)

The sandblasting and acid etching (SLA) method help to create a rough topography for mechanical fixation, and long-term stability, but it won't give early bone healing. The plasma treatments will not change the topography, and gives hydrophilicity(Chou et al., 2017). SLA is a surface modification, which will induce surface roughening by applying strong acid on to the blasted surface of implant. It is a combined method of blasting the surface with large grit sand particles and giving a sequential acid etching. This method will induce micro pits and homologous roughening to increase the cell adhesion which will improve the osseointegration property of the implant. Many study has shown the evidences that the SLA treated Ti implant was useful for the improvement in osseointegration, and cell adhesion (Mei et al., 2018).

With this background the present study was aimed to evaluate two different types of surface modified dental implants in a rabbit model after long term implantation.

1.2.5 BONE SPECIAL STAINS:

Special stain can be referred as those stains which are used for a special purpose and it is not used routinely.

1.2.5.1 Stevenel's Blue:

Stevenel's Blue is a reliable polychromatic stain for thin resin embedded tissue sections .It is a time saving procedure. The staining solution was early used to stain human parasites by L. Stevenel (1918). The stain helps to differentiate nuclear, cytoplasm, and extracellular components. Stained section are resistant to time-fading because of the stable bond between Stevenel's Blue and the tissues(del Cerro et al., 1980).

1.2.5.2 Van gieson picrofuschin stain:

Van Gieson's stain is a mixture of acid fuchsin and picric acid. It is the simple stain that used for differential staining of collagen and other connective tissue. It was

introduced in histological technique by histology by Ira Van Gieson (Bozhkov et al., 2017).

1.2.6 BONE HISTOMORPHOMETRY

It is a quantitative technique for the bone tissue. By this method the amount of bone tissue distribution can be analyzed. In bone histomorphometry the bone to implant contact percentage (BIC) is calculated, the increased percentage of the BIC indicates the increase in bone remodelling. Bone histomorphometry helps to analyze the efficacy of an implant and analyze the safety and effiency of new treatment approach(Rauch, 2014).

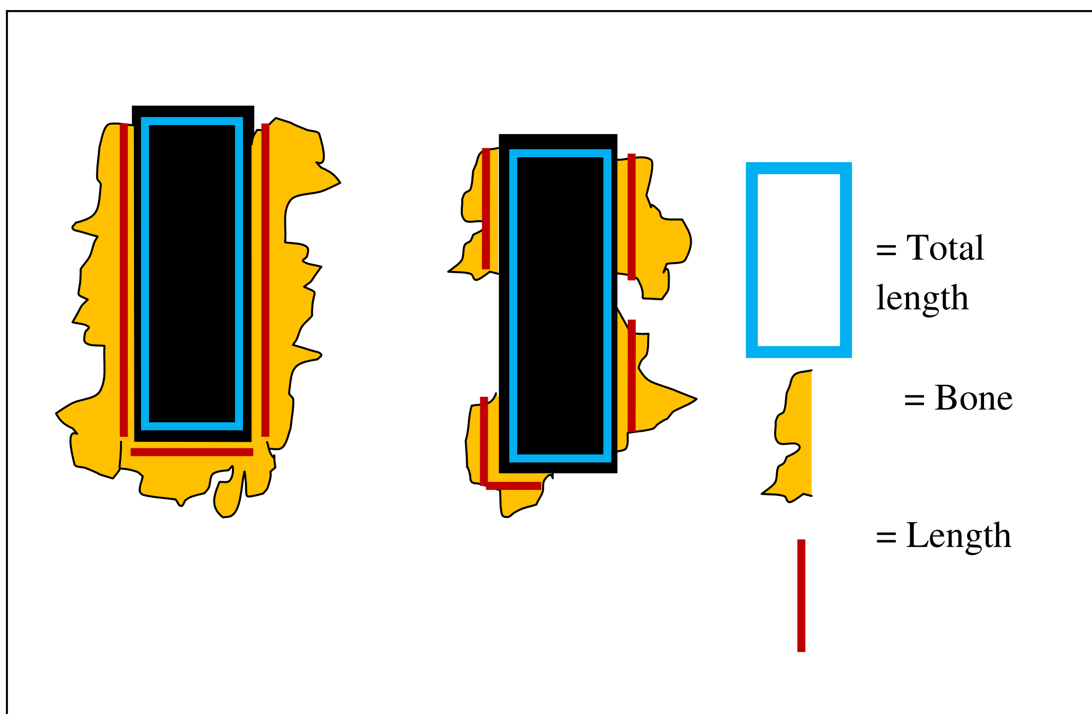


Figure: 5 Histomorphometry analysis-Bones to implant contact

1.3HYPOTHESIS

Surface modification treatment by acid etching in Ti implant surface promotes osseointegration

1.4AIM

To evaluate the osseointegration of acid etched Ti implants in rabbit model

1.5 OBJECTIVES

- Gross and Histopathological analysis of osseointegration of surface treated implants in rabbit femur bone
- Histomorphometric analysis to calculate the percentage of bone material contact
- Quantitative analysis of osteogenic and associated gene expression study using RT-PCR technique

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 MATERIALS USED FOR STUDY

2.1. 1 Animal model used

New Zealand White rabbit were used as the animal model for this study. Twelve Adult New Zealand white rabbit, aged more than 10 months having body weight more than 2.5kg of both sexes were selected for the study. The study was approved by Institutional Animal Ethics Committee (IEC) (B form number: SCT/IAEC/275/APR/2018/96).

2.2 *IN VIVO LONG TERM EVALUATION*

The study protocol were based on the ISO 10993-6 standards. Rabbit Femur condyle region was implanted with surface treated titanium dental screws, each hind limb received a test and control implant randomly. After 6 months of implantation, animals were euthanized humanely by the intravenous injection of Thiopental sodium (high dose).

2.3 SAMPLE COLLECTION

Femur bone with implant was collected. Implant with surrounding bone tissue as well as normal bone tissue were collected. For molecular studies the sample bone tissue was stored in a falcon tube contained RNAlater^{TM}. For Histological studies the bone tissue samples collected were stored in 10% Neutral Buffer Formalin (NBF).

2.4 HISTOLOGICAL ANALYSIS

2.4.1 Instruments used

Vacuum pump (LabCONCO, USA), High Precision saw microtome (Accutom100, Struers, Denmark), Grinder cum polisher (Ecomet 3, Buhler, USA), Water bath (HI 1210, LABINDIA), Light microscope (Nikon E 600, Japan), Trinocular microscope (Nikon Eclipse Ni, Japan) with camera (Nikon Ri1).

2.4.2 Chemicals and Reagents

10% Neutral Buffer Formaldehyde (NBF), Alcohol (70%,80%,96% and absolute), Acetone (Thermo Fischer, Mumbai), Methyl Methacrylate (MMA), Benzoyl peroxide (Merck, Mumbai), 5% Sodium hydroxide, Potassium permanganate (Merck, Mumbai), Methylene blue (Qualigen,Mumbai), Distilled water, Cyano acrylate glue (Alteco chemicals, Japan), Stevenel's blue and Van Gieson's Picrofuschin stain.

2.4.3 Processing and embedding of Bone with implant

For sequential matching with the hardness of bone and permitting undisrupted sections, MMA resin was used. Bone tissue collected and stored in 10%NBF for 2 days for fixation.

Following was the protocol for tissue processing

70% Alcohol for 4 days

80% Alcohol for 4 days

96% Alcohol for 4 days

100% Alcohol for 2 days

Alcohol: Acetone (1:1,v/v) mix for 1day

Dehydrated tissue bocks were infiltrated by using washed Methyl Methacrylate (MMA) monomer then kept in refrigerator for 4 days

2.4.4 Tissue embedding in PMMA

Freshly prepared embedding solution were used, each tissue were embedded in separate clean bottles (size of the bottle depends on the size of tissue). . Embedding solution was poured in to a clean bottle, and labelled each bottle with identification number. Using clean sterile forceps the bone tissue is placed in the embedding solution with cutting surface faces down. This will ensure that the cut can be made in the desired surface. Placed the cap loosely in the bottle and bottle were kept in

vacuum desiccator to remove any air bubbles trapped. Released the vacuum slowly and tightened the cap carefully, then kept the bottle in vacuum desiccator for 2-3 days. Hard resin texture indicated the completion of polymerization.

2.4.5 Section cutting

Precision saw microtome (Accutom 100, Denmark) was used for getting thin sections of resin embedded tissue (100µm). Sectioning of the implant site was done. Each section was carefully taken out for further process. Sequentially each section was fixed on clean glass slide using cyanoacrylate glue.

2.4.6 Grinding and polishing

For clear microscopic vision tissue section has to be grinded and polished using Grinder cum polishing machine (Ecomet 3, USA) using different sized grit paper and finally polished using a muslin cloth.

2.4.7 Staining of resin embedded tissue section

- **Preparation of Stevenel's Blue stain**

Potassium permanganate solution was prepared by using dissolving 1.5g Potassium permanganate in 75ml distilled water. To this solution methylene blue solution (1g dissolved in distilled water) was added. The mixture was then kept in boiling water bath until the precipitates were dissolved. After the solution reached the room temperature it was filtered and kept in refrigerator.

- **Preparation of Van Gieson Picrofuschin stain**

1% Acid fuschin was prepared by dissolving 0.1g acid fuschin in 10ml distilled water. To the 1% acid fuschin solution 100ml of saturated picric acid was added and mixed well. The mixture was kept in reagent bottle and labelled.

Staining

Resin embedded thin sections were then stained using Stevenel's blue stain in pre heated water bath for 5 minutes then removed the excess stain by dipping in distilled water which was kept in boiling water bath. These resin sections were then counter stained with Van Gieson Picrofuschin stain for 3 minutes at room temperature. Excess stain was removed.

2.4.8 Microscopic evaluation

Stained sections mounted on glass slides were observed under a bright field trinocular microscope (NikonNiE, Japan) and microphotographs were captured using the Camera (Nikon Ri1).

2.5 HISTOMORPHOMETRY ANALYSIS

Using the Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) software, the photomicrographs of bone with implant were analysed and using the following formula bone to implant contact percentage was calculated for both test and control implant sites.

$$\text{BIC\%} = \frac{\text{Total length of bone contact with implant}}{\text{Total length of implant}} \times 100$$

Han J, Hong G, Lin H, et al. Biomechanical and histological evaluation of the osseointegration capacity of two types of zirconia implant. International Journal of Nanomedicine. 2016

2.6 MOLECULAR STUDIES

2.6.1 RNA isolation

Instruments

PCR cabinet (imset^{TM}), High speed refrigerated Centrifuge 5417R (Eppendorf, USA), Tissue homogenizer (Polytron), Spectrophotometer (Nano Drop Inc. Wilminton, USA)

Chemicals

RNAlater^{TM}(Thermo fischer,USA), Absolute ethanol(Himedia, Mumbai) Chloroform(Ranbaxy, New Delhi), Iso-propanol(Merck, Mumbai), water Ultra Pure ^{TM}(Thermo fischer,USA), Distilled water(Life Technologies, USA), Liquid Nitrogen, TRIzol^{R}.

For total RNA isolation from the collected bone samples TRIzol extraction method was used. Sample stored in -70^{0}C in RNAlater were thawed to room temperature. Bone tissue sample taken out from RNAlater and washed using DEPC treated water. Washed samples were then crushed using mortar and pestle by adding liquid nitrogen while kept in cold dry ice. Powdered samples were collected in sterile eppendorf tube.

RNA isolation include six steps

- **Homogenization:**

Added 1ml TRIzol into 0.1g bone powder in a sterile 2 ml eppendorf tube. The content was then homogenized using homogenizer at room. After homogenization the sample was incubated for 5 minutes at room temperature followed by centrifugation at 1500rpm for 4 minutes at 4^{0}C then supernatant was collected into a new eppendorf tube.

- **Phase separation**

To the collected supernatant, added chloroform and mixed well. Incubated the mixture at room temperature, for about 5 minutes. After incubation, the content was centrifuged at 12000rpm for 15 minutes at 4^{0}C, the content will be separated into three layers.

Liquid top layer (colourless layer) - contains RNA

Semisolid middle layer –Mostly DNA

Bottom layer (Red colour) - Organic solvents and protein

- **RNA Precipitation**

Carefully transferred the colourless liquid top layer into a fresh eppendorf tube. Added equal amount or 0.5 times more isopropanol into the top layer. Mixed gently by inverting the tube. The mixture was then kept at room temperature for 10 minutes. Centrifuged the mixture at 12000rpm at 4^0C for 10 minutes, Supernatant was discarded and pellet was collected

- **RNA precipitate cleaning**

Added 1ml of 75% ethanol into the pellet and vortexed gently. The content was centrifuged at 7500rpm at 4^0C for 5 minutes. Discarded the supernatant and pellet was collected.

- **Dissolving RNA**

Kept the tube which contained the pellet open for few minutes to dry the precipitate. Added 25µl DEPC treated water and mixed the content gently. Kept in ice for 10-15 minutes.

- **Analysis of RNA purity and quantification**

The RNA purity of total RNA isolated was checked at OD 260/280 nm and quantified at OD 260nm by using Nanodrop spectrophotometer.

2.6.2 cDNA synthesis

Materials: Reverse Transcriptase Core kit (RT-RTCK-03, Eurogentec), RNase free water, Thermal cycler (eppendorf, USA), PCR tubes.

Reagent	Volume(µl)
10x reaction buffer	1
25Mm $MgCl_2$	2
2.5Mm dNTP	2
oligo d(T)15VN	0.5
RNAse inhibitor	0.2
Euro Script RT	0.25
RNAse free water	As required
Template	As needed with respect to the RNA purity
Total volume	10

Table 3: Master mix protocol for cDNA synthesis for 10µl

- Prior to mixing thawed all the reagents and spin the reagents.
- After mixing the content was kept in thermal cycler for cDNA synthesis.

Reaction conditions in PCR for cDNA synthesis.

- Preheat the lid at 105^0C
- Annealing- 25^0C for 5minutes
- Extension- 42^0C for 1hr

The cDNA synthesised was stored at -20^0C.

2.6.3 Real Time PCR Amplification and quantification of COL1 A1, SPP 1, SPARC, RUNX 2, GAPDH, VEGF, TGF-β gene expression

- **Materials used**

Takyon™ No Rox SYBR® MasterMix dTTP Blue, forward and reverse Primers Thermal cycler (qTower 3, Analytik Jena, Germany), cDNA of individual samples.

Sl. No	Oligoname	5'-----------3' Sequence	Base pairs length
1	Collagen 1 forward	GCAAGAACGGAGATGACGGA	20
	Collagen 1reverse	TTGGCACCATCCAAACCACT	20
2	RUNX 2 forward	ACCAGTCTTACCCCTCTTACCT	22
	RUNX 2 reverse	AGGTGCTGGGCTCTGAATCTG	21
3	SPARC forward	GAAGTAGTGGCCGAAAACCC	20
	SPARC reverse	TGGGGGTGTTGTTCTCATCC	20
4	GAPDH forward	CAACGAATTTGGCTACAGCA	20
	GAPDH reverse	AAACTGTGAAGAGGGGCAGA	20
6	TGF-β forward	CTGGAACGGGCTCAACATCT	20
	TGF-β reverse	CAGCAAAGGACAGGTCTCCA	20
7	VEGF forward	CTTGCAGATGTGACAAGCCG	20
	VEGF reverse	AGTCTTTCCCGGTGAGAGGT	20
8	SPP 1 forward	CGATGACTCTCACCACTCCG	20
	SPP 1 reverse	CTGCGAAATTCACGGCTCTG	20

Table: 4 list of primers for gene expression study

Reagent	Volume in µl
Takyon master mix	10
Forward primer	0.6
Reverse primer	0.6
Nuclease free water	6.3
cDNA	2.5
Total volume	20

Table 5: RT-PCR master mix protocol

After thawing cDNA and reagents mix the contents accordance with the above protocol. Mix well the content.

Reaction mixture incubated in the following condition.

- Enzyme inactivation – 95^0C for 3 minutes
- Denaturation - 95^0C for 1-3 seconds

- Annealing - 60^0C for 20 seconds
- Extension - 72^0C for 30 seconds

2.7 RT-PCR analysis

The cDNA copy number of target gene was normalized with the reference gene, GAPDH (house keeping gene). Real time PCR gene expression measurement were converted to delta Ct value and then relative gene expression were calculated using delta-delta Ct method,(Livak and Schmittgen, 2001)

$$\Delta Ct = Ct \text{ of target gene} - ct \text{ of reference gene}$$
$$\Delta\Delta Ct = \Delta Ct \text{ of test sample} - \Delta Ct \text{ of control sample}$$
$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25 4, 402–408. doi: 10.1006/meth.2001.1262

2.8 STATISTICAL ANALYSIS

Data observed from the molecular gene expression analysis and the histomorphometric analysis was calculated using the statistical analysis was carried out for all the quantitative data. Student t-test was used to determine P value so that the presence of significant difference can be found out. P value<0.05 is considered as significant. All the values given in table are represented by Mean ±SD.

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1 *IN VIVO EVALUATION*

All animals were found healthy and no limping was observed during the experimental period. All the rabbits presented satisfactory postoperative results such as wound healing, with no evidence of inflammation or infection at the surgical site.

3.2 *GROSS PATHOLOGY OBSERVATION*

All implants were found at the implant site. No gross abnormalities were detected at the implant site. The implant surfaces were covered with a thin layer of connective tissue on the periosteal surface.

3.3 *HISTOPATHOLOGY OBSERVATION*

On microscopic evaluation in test and control implant site, absence of necrosis, inflammation and degeneration at implant-bone interface was observed. No intervening soft tissue between the implant and bone was observed. New bone formation was observed at the implant interface. New woven bone was observed which was found filling the interface. Osteoblasts cells were observed in the new bone region at multiple focal areas. The Titanium screw's threads were observed to be anchoring to the new bone at the test implant. In control implant multiple areas of implant surface not covered with new bone were observed in comparison to test implant surface. Implants were observed without any gross abnormalities like budging, fluids collection and breaks in implant bone interface. Histologically, no qualitative difference in the new bone formation could be noted between control (SLA) and test (acid etched Ti) implants.

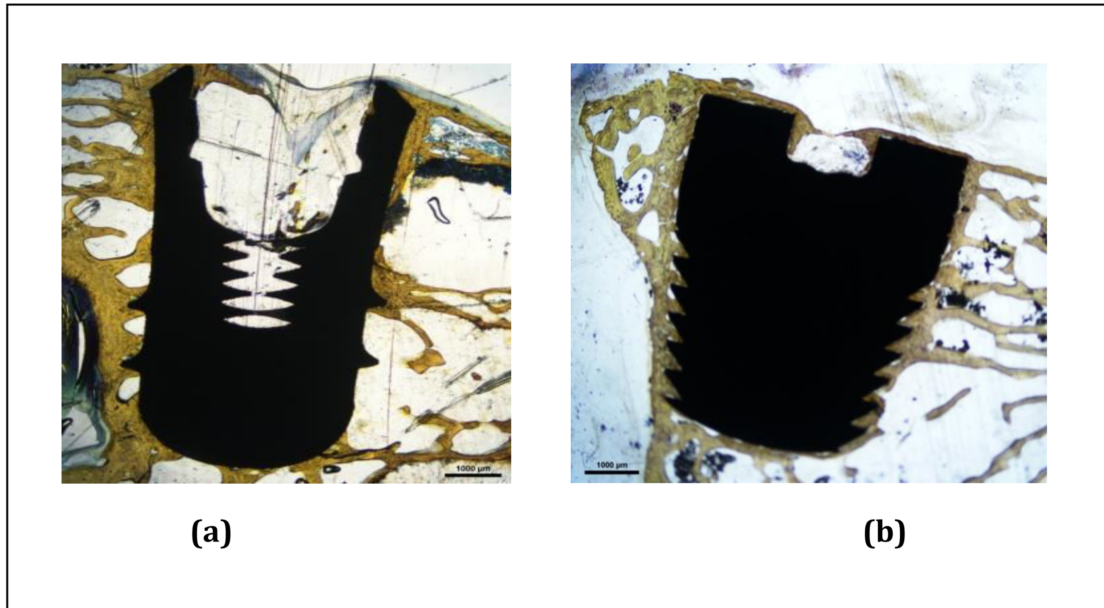


Figure 6: Femur cross section- Stevenel's Blue - Van Gieson Picrofuschin stained microscopic images of the bone with implant interface (**a**)Control-SLA treated Ti implant (**b**) Test-Acid etched Ti implant, (1X magnification, scale bar: 1000µm)

New woven bone formation without any intervening soft tissue was observed at implant interface of both control and test. Acid etched Ti (Test) implant showed new woven bone formation at the implant interface without any discontinuation which indicated that the acid etched Ti (Test) implant promoted the new bone formation and has good osseogenic property compared to the control implant. Qualitative analysis of osseointegration revealed that the surface treatment by acid etching promoted new bone formation(Velasco-Ortega et al., 2019).

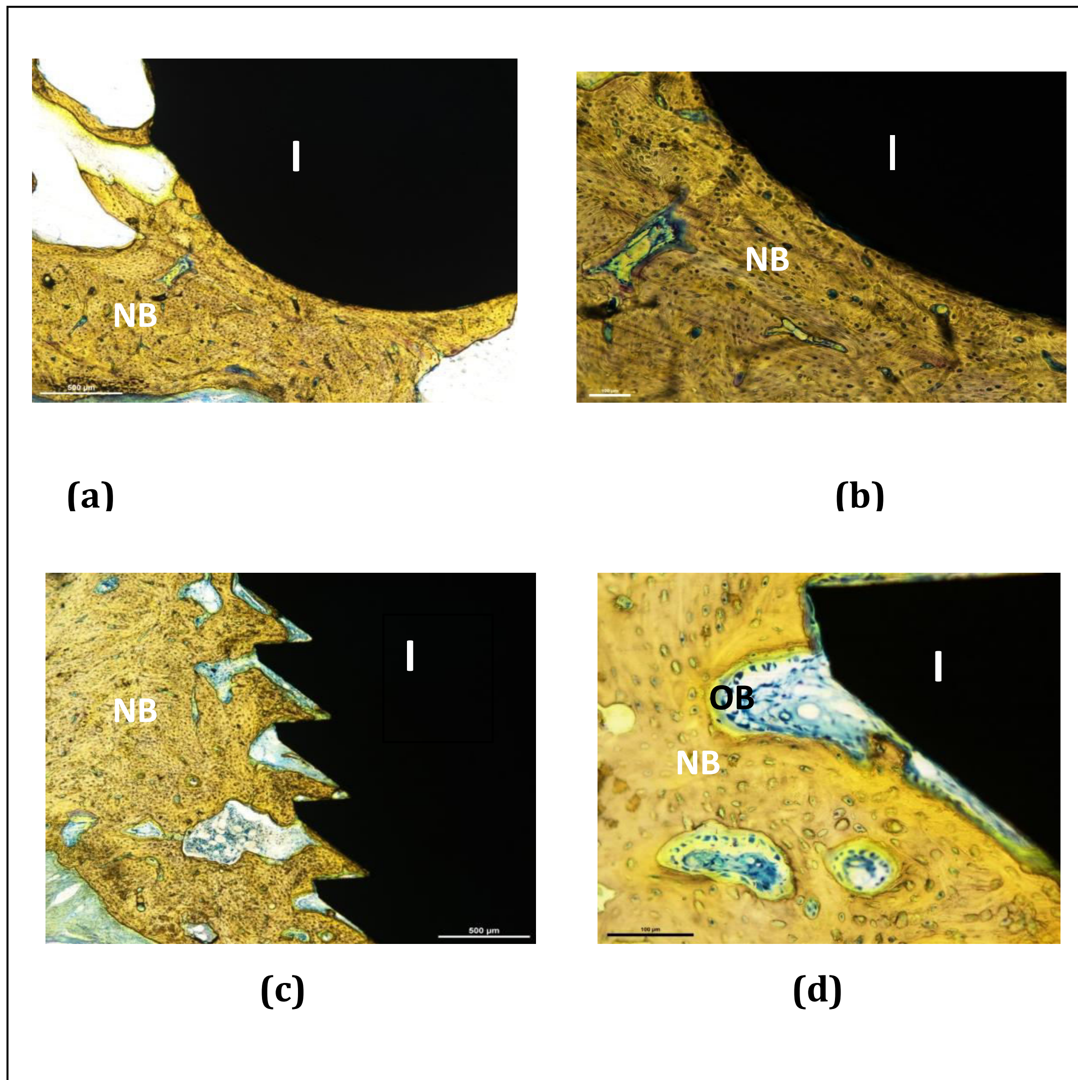


Figure: 7 Femur cross section- Stevenel's Blue - Van Gieson Picrofuschin stained microscopic images of the bone with implant interface (**a**)Control-SLA treated Ti implant 4X magnification (scale bar: 500µm) (**b**) Control-SLA treated Ti implant 10X magnification (scale bar: 100µm) (**c**)Test-Acid etched Ti implant, 4X magnification (scale bar: 500µm) (**d**)- Test-Acid etched Ti implant, 10X magnification (scale bar: 100µm) New woven bone formation with gaps, *I-implant, NB-new bone, OB- Osteoblasts.*

Acid etched Ti implant has shown improved osseointegration, in higher magnification new woven bone formation attached to the implant surface could be clearly noted. No intervening soft tissues were observed between implant interface and bone. New woven bone formation was observed continuously along the implant

interface in acid etched Ti implant. Osteoblast activity is observed in both test and control. The surface modification enhanced homogenous roughening, which resulted in the cell proliferation enhancement and accelerated the osseointegration (Velasco-Ortega et al., 2019).

3.4 HISTOMORPHOMETRIC ANALYSIS

Bone to implant contact percentage

The maximum bone implant contact was observed in Ti implant treated with Acid etching compared to SLA Ti implant

$$BIC\% = \frac{Total\ length\ of\ bone\ contact\ with\ implant}{Total\ length\ of\ implant} \times 100$$

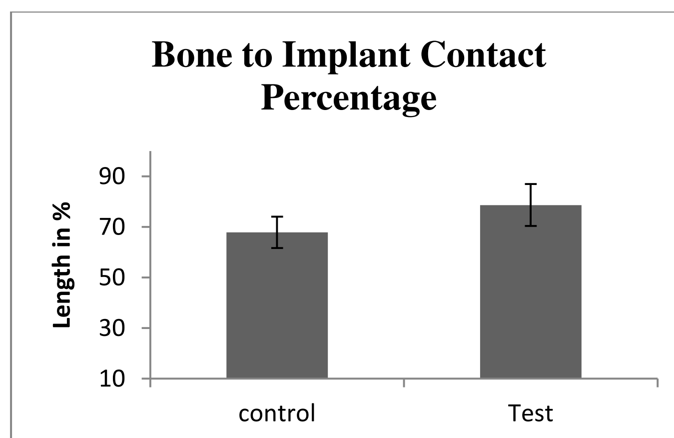


Figure 8: Graphical representation of Percentage of Bone to implant contact in Femur, values is represented in Mean ±SD (n=4)

The control implant has shown 67.85% of new bone formation in contact with the implant. Test implant has shown 78.65% of new bone formation.

The quantitative analysis of new woven bone formation across the implant interface showed an increase in test group when compared with the control group. Homogenous roughening in implant surface mediates the better cell adhesion thus the osseointegration is promoted in test group. A study conducted on the osteointegrative ability of acid etching on titanium alloys, observed that the higher bone to implant contact in acid etched titanium alloy(Granato et al., 2019).

3.5 MOLECULAR STUDIES

3.5.1 GENE EXPRESSION PATTERN

The expression of osseogenic genes (COL1A1, RUNX2, and SPARC and SPP1), Angiogenic gene (VEGF) and TGF-β, were quantitatively determined using RT-PCR, and fold change was recorded. GAPDH gene was used as the reference gene. Gene expression of test and control samples was normalized with the normal bone sample.

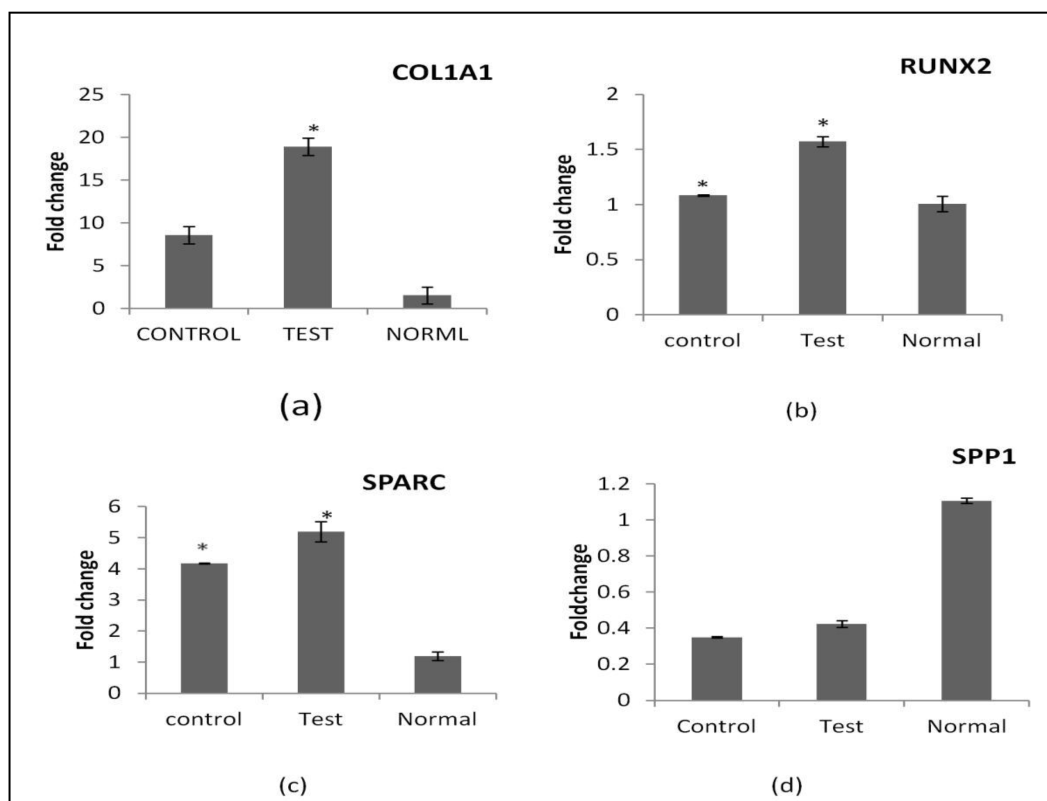


Figure 10: **Graphical representation of RT-PCR data-COL1A1, RUNX2, SPARC and SPP1,** depicting the fold change in expression values are represented in Mean ±SD (n=3). P value <0.05(significant value compared with non grouped normal bone)(a) COL1A1 (b)RUNX2 (c) SPARC (d)SPP1(*Abbreviation:COL1A1-Collagen type I alpha 1,RUNX2-Runt-related transcription factor 2, SPARC-secreted protein acidic and rich in cysteine,SPP1-Secreted phosphoprotein 1)*

The expression of COL1A1, RUNX2 and SPARC genes were found to be up regulated in acid etched Ti implant. The osteopontin, SPP1 was found to be down

regulated. The up regulated expression of COL1A1, RUNX2, and SPARC indicates the bone was under mineralization phase. The main structural protein, COL1A1 was the major component in organic phase in bone. They were actively participitated in the bone mineralization phase. Bai et al., 2020 has reported that the COL1A1 gene expression increased while studying biomimentic osteogenic peptide in chronic inflammatory patients. The COL1A1 expression along with other genes indicated the osseogenesis. RUNX2 gene transfer study in MSCs had identified that the Runx2 gene has osseogenic activity and the osseogenic activity has been enhanced by the adenoviral transfer of RUNX2 gene in MSCs(Zhao et al., 2005). Delany and Hankenson in 2009 studied the gene expression of SPARC in the bone mineralization and proliferation, SPARC gene expression revealed the role of SPARC in remodeling process of bone. They have shown a wider pattern of activity in both mineralized and non mineralized tissue (Rosset and Bradshaw, 2016).

SPP1 gene found to be down regulated in both control and test, and expressed in normal tissue. The SPP1 down regulation in tissue with surface treated implants is indicating that the bone is under mineralization. Osteopontin is a non collagenous protein present in the organic phase of bone mainly in the mineralized bone matrix(Singh et al., 2018).The high expression of SPP1 in normal bone is due to the mineralized bone matrix in normal bone compared to the surface treated implants. SPP1 has role in bone metabolism and they are mainly seen in the mineralized tissue. A bone niche recapitulation study on rabbit model conducted Minardi et al., in 2019 observed the SPP1 gene expression in mineralized bone along with other osteogenic markers (Minardi et al., 2019).

Foster et al.,2018, had studied the osteopontin(SPP1) effect in dental mineralization and they reported that the SPP1has been expressed in the mineralized bone in early osteoblasts during periodontal tissue formation(Foster et al., 2018)

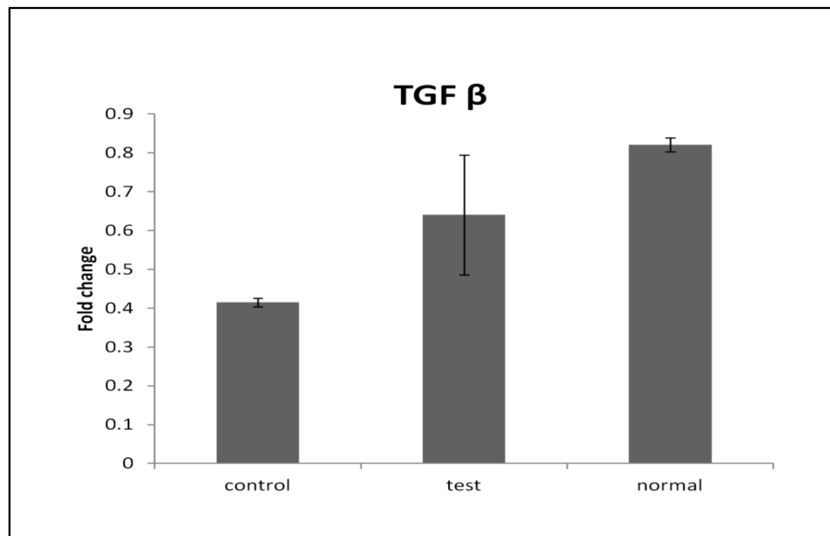


Figure 11: Graphical representation of RT-PCR data-TGF β, depicting the fold change in expression of TGF-β gene, values is represented in ±SD (n=3). P value >0.05, (*Abbreviation: TGF-β Transforming growth factor beta).*

The expression of TGF-β was found to be down regulated in (Test) acid etched Ti implant compared to normal bone, which indicated that the bone healing was occurring and TGF- β was secreted by osteoblast cells (Abou-Ezzi et al., 2019).In 2019 study conducted by Abou-Ezzi et al on the TGF-β, provides the information about the TGF-β role in osseogenesis and bone healing. The pharmacologic inhibition of the TGF-b-receptor I kinase using SD-208 increases trabecular bone mass through stimulating osteoblastic bone formation and repressing osteoclastic resorption. During bone healing the TGF β expressed and mediated the expression other inflammatory markers. TGF β treatment supported the PLR (platelet-lymphocyte ratio) activation in osteocytes (Abou-Ezzi et al., 2019). Activation of TGF β promote MSCs production for remodelling of bone TGF β has role in bone remodelling during which they recruited from the bone matrix. TGF β has important role in bone resorption regulation, Dole et al in 2017 reported that the TGF β mutation reduces the PLR activation and reduced the osteoclast formation in bone resorption (Dole et al., 2017). The TGF β expression in normal bone was noted in this which may be due to the signalling cascade.

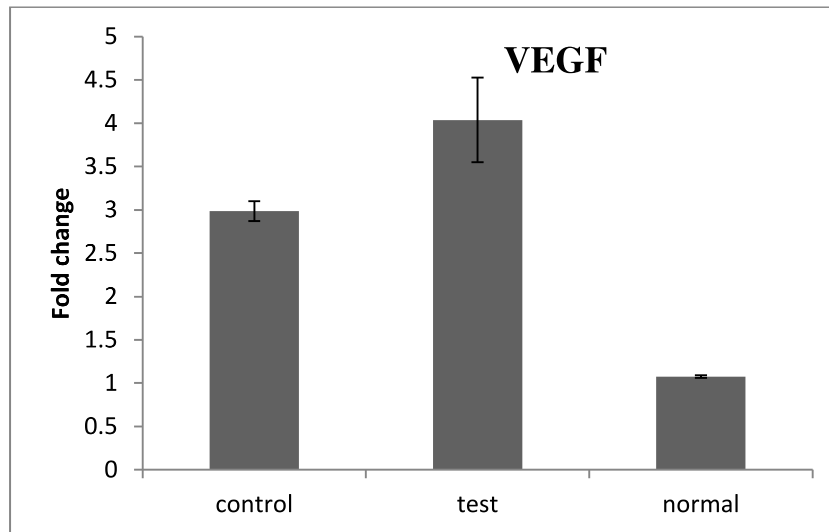


Figure 12: **Graphical representation of RT-PCR data- VEGF**, depicting the fold change in expression of VEGF gene (values are represented in Mean ±SD (n=3). P value <0.05, *(Abbreviation: VEGF-Vascular endothelial growth factor).*

The expression of VEGF was found to be up regulated in acid etched Ti implant. VEGF promotes the new blood vessel formation in and around the newly formed bone. VEGF has a major role in coupling angiogenesis and osseogenesis. Izquierdo-Barba et al, in 2019 studied the effect of VEGF absorbed Ti scaffold in osteoporototic sheep. It has been observed that the VEGF scaffold had promoted the angiogenesis and had shown an improved effect on ossification(Izquierdo-Barba et al., 2019).

CHAPTER 4

SUMMARY, CONCLUSION AND FUTURE PROSPECTS

4.1 SUMMARY

The present work was done to investigate the osseointegration of two different types of surface treated implants in New Zealand white rabbit model. The implantation was done on the femur condyle side with SLA treated Ti dental screw type implant and acid etched Ti screw implant. The osseointegration ability of surface treated implants was compared with each other. Grossly, no abnormality could be observed at the implant sites. Histopathological analysis revealed no inflammation, necrosis and degeneration of tissue at the implant site in both groups, which indicates that the materials were biocompatible. New woven bone formation was observed at the implant interface without any intervening soft tissue in both groups. Osteoblast cell activity was observed in both control and test implant site in the new bone region, which indicates that both the implant has favoured osseointegration Quantitative analysis of new bone formation was analysed by histomorphometric method by calculating the bone to implant contact surface area from the captured images using ImageJ software. It was observed that the test group showed increased new woven bone formation compared to the control group. Acid etched Ti implant has shown new woven formation throughout the implant interface without any discontinuity supporting the osseointegration ability of the implant. From the molecular gene expression analysis it was found that osseogenic genes (COL1A1, RUNX2 and SPARC) have shown up regulated activity in the surface treated implants. Angiogenic marker, VEGF gene expression has found to be up regulated in the surface treated Ti. SPP1 gene has found to be down regulated in the surface treated Ti implants. TGF β has found to be down regulated in the surface treated Ti implants. The up regulated gene expression of osteogenic markers (COL1A1, RUNX2 and SPARC) indicates that the surface treated implant has promoted bone healing and bone mineralization which reveals that the osseogenis has promoted in surface treated implants. Late phase marker SPP1 down regulated gene expression in surface treated implants indicates the bone mineralization phase and osseogenic differentiation, SPP1 is actively expressed in mineralized bone matrix of normal bone. The significant up regulated expression of VEGF indicates the new blood vessel formation in surface treated implants; it also reveals the role of VEGF in

osseogenesis and angiogenesis coupling. TGF β found to be down regulated in the surface treated implants, which indicates that the bone remodelling, although the TGF β expression in normal bone was also noted. The results indicated that both the surface treated Ti implants favoured osseointegration whereas the acid etched Ti implant showed improved osseogenic gene expression compared to the SLA treated implant.

4.2 CONCLUSION

The results of the gross and histopathological evaluation, histomorphometry and gene expression for osseogenic, angiogenic and inflammatory marker in the present study indicated that the acid etched implant is biocompatible and has good osseointegration property after long-term implantation in a rabbit femur model.

4.3 FUTURE PROSPECTS

- To elucidate the role of microRNA involved in the osseointegration when implanted with Surface treated Ti implants
- To study the proteins involved in the osseointegration by immunological analysis.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Abdelrahim, R.A., Badr, N.A., Baroudi, K., 2016. The effect of plasma surface treatment on the bioactivity of titanium implant materials (in vitro). J Int Soc Prev Community Dent 6 1 , 15–21. doi:10.4103/2231-0762.171592
2. Abou-Ezzi, G., Supakorndej, T., Zhang, J., Anthony, B., Krambs, J., Celik, H., Karpova, D., Craft, C.S., Link, D.C., 2019. TGF-β Signaling Plays an Essential Role in the Lineage Specification of Mesenchymal Stem/Progenitor Cells in Fetal Bone Marrow. Stem Cell Reports 13 1 , 48–60. doi:10.1016/j.stemcr.2019.05.017
3. Abrahamsson, I., Berglundh, T., Linder, E., Lang, N.P., Lindhe, J., 2004. Early bone formation adjacent to rough and turned endosseous implant surfaces. An experimental study in the dog. Clin Oral Implants Res 15 4 , 381–392. doi10.1111/j.1600-0501.2004.01082
4. Aita, H., Hori, N., Takeuchi, M., Suzuki, T., Yamada, M., Anpo, M., Ogawa, T., 2009. The effect of ultraviolet functionalization of titanium on integration with bone. Biomaterials 30 6 , 1015–1025. doi:10.1016/j.biomaterials.2008.11.004
5. Akiyama, H., Chaboissier, M.-C., Martin, J.F., Schedl, A., de Crombrugghe, B., 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 16 21 , 2813–2828. doi:10.1101/gad.1017802
6. Albrektsson, T., Wennerberg, A., 2004. Oral implant surfaces: Part 1--review focusing on topographic and chemical properties of different surfaces and in vivo responses to them. Int J Prosthodont 17 5 , 536–543.
7. Anusuya, G.S., Kandasamy, M., Jacob Raja, S.A., Sabarinathan, S., Ravishankar, P., Kandhasamy, B., 2016. Bone morphogenetic proteins: Signaling periodontal bone regeneration and repair. J Pharm Bioallied Sci 8 Suppl 1 , S39–S41. doi:10.4103/0975-7406.191964
8. Ao, H., Zong, J., Nie, Y., Wan, Y., Zheng, X., 2017. An in vivo study on the effect of coating stability on osteointegration performance of collagen/hyaluronic acid multilayer modified titanium implants. Bioact Mater 3 1 , 97–101. doi:10.1016/j.bioactmat.2017.07.004
9. Artas, G., Gul, M., Acikan, I., Kirtay, M., Bozoglan, A., Simsek, S., Yaman, F., Dundar, S., Artas, G., Gul, M., Acikan, I., Kirtay, M., Bozoglan, A., Simsek, S., Yaman, F., Dundar, S., 2018. A comparison of different bone graft materials in peri-implant guided bone regeneration. Brazilian Oral Research 32. doi:10.1590/1807-3107bor-2018.vol32.0059
10. Baek, S.M., Polyakov, A.V., Moon, J.H., Semenova, I.P., Valiev, R.Z., Kim, H.S., 2017. Effect of surface etching on the tensile behavior of coarse- and ultrafine-grained pure titanium. Materials Science and Engineering: A 707, 337–343. doi:10.1016/j.msea.2017.09.065
11. Bai, J., Wang, H., Chen, H., Ge, G., Wang, M., Gao, A., Tong, L., Xu, Y., Yang, H., Pan, G., Chu, P.K., Geng, D., 2020. Biomimetic osteogenic peptide with mussel adhesion and osteoimmunomodulatory functions to ameliorate interfacial osseointegration under chronic inflammation. Biomaterials 255, 120197. doi:10.1016/j.biomaterials.2020.120197

12. Ban, S., Iwaya, Y., Kono, H., Sato, H., 2006. Surface modification of titanium by etching in concentrated sulfuric acid. Dent Mater 22 12 , 1115–1120. doi:10.1016/j.dental.2005.09.007
13. Berebichez-Fridman, R., Montero-Olvera, P., Gómez-García, R., Berebichez-Fastlicht, E., 2017. An intramedullary nail coated with antibiotic and growth factor nanoparticles: An individualized state-of-the-art treatment for chronic osteomyelitis with bone defects. Med. Hypotheses 105, 63–68. doi:10.1016/j.mehy.2017.06.023
14. Blázquez-Medela, A.M., Jumabay, M., Boström, K.I., 2019. Beyond the Bone: Bone Morphogenetic Protein (BMP) Signaling in Adipose Tissue. Obes Rev 20 5 , 648–658. doi:10.1111/obr.12822
15. Bone Classification and Structure | Anatomy and Physiology n.d. URL https://courses.lumenlearning.com/nemcc-ap/chapter/bone-classification/#m46282-fs-id1170296
16. Bone Morphogenetic Proteins: Growth Factors: Vol 22, No 4 n.d. URL https://www.tandfonline.com/doi/abs/10.1080/08977190412331279890
17. Bone Regeneration. Translational Regenerative Medicine, 313–333 | 10.1016/b978-0-12-410396-2.00024-4 URL https://scihub.wikicn.top/https://doi.org/10.1016/B978-0-12-410396-2.00024-4
18. Bone Remodeling and Repair 2018. Biology LibreTexts. URLhttps://bio.libretexts.org/Bookshelves/Introductory_and_General_Biology/Book%3A_General_Biology_(Boundless)/38%3A_The_Musculoskeletal_System/38.2%3A_Bone/38.2E%3A_Bone_Remodeling_and_Repair
19. Bone Structure - an overview | ScienceDirect Topics , n.d. URL https://www.sciencedirect.com/topics/materials-science/bone-structure
20. Bonewald, L.F., 2010. Chapter 313 - Cell–Cell and Cell–Matrix Interactions in Bone, in: Bradshaw, R.A., Dennis, E.A. (Eds.), Handbook of Cell Signaling (Second Edition). Academic Press, San Diego, pp. 2647–2662. doi:10.1016/B978-0-12-374145-5.00313-2
21. Bozhkov, A.I., Klimova, O.M., Nikitchenko, Y.V., Kurguzova, N.I., Linkevych, O.S., M. Lebid, K., Protsenko, O.S., Remneva, N.A., Al-Bahadly, A.M.M., Al-Begai, M.A.Y., 2017. Ontogenetic Approach to the Study of Mechanisms of Copper-Induced Liver Fibrosis. AAR 06 03 , 39–54. doi:10.4236/aar.2017.63005
22. Buser, D., Broggini, N., Wieland, M., Schenk, R.K., Denzer, A.J., Cochran, D.L., Hoffmann, B., Lussi, A., Steinemann, S.G., 2004. Enhanced Bone Apposition to a Chemically Modified SLA Titanium Surface. J Dent Res 83 7 , 529–533. doi:10.1177/154405910408300704
23. Casarrubios, L., Gómez-Cerezo, N., Sánchez-Salcedo, S., Feito, M.J., Serrano, M.C., Saiz-Pardo, M., Ortega, L., de Pablo, D., Díaz-Güemes, I., Fernández-Tomé, B., Enciso, S., Sánchez-Margallo, F.M., Portolés, M.T., Arcos, D., Vallet-Regí, M., 2020. Silicon substituted hydroxyapatite/VEGF scaffolds stimulate bone regeneration in osteoporotic sheep. Acta Biomaterialia 101, 544–553. doi:10.1016/j.actbio.2019.10.033

24. Chaparro, O., Linero, I., 2016. Regenerative Medicine: A New Paradigm in Bone Regeneration, in: Zorzi, A.R., de Miranda, J.B. (Eds.), Advanced Techniques in Bone Regeneration. InTech. doi:10.5772/62523
25. Chen, D., Zhao, M., Mundy, G.R., 2004. Bone morphogenetic proteins. Growth Factors 22 4 , 233–241. doi:10.1080/08977190412331279890
26. Chen, X., Wang, Z., Duan, N., Zhu, G., Schwarz, E.M., Xie, C., 2018. Osteoblast-Osteoclast Interactions. Connect Tissue Res 59 2 , 99–107. doi:10.1080/03008207.2017.1290085
27. Chevallier, N., Anagnostou, F., Zilber, S., Bodivit, G., Maurin, S., Barrault, A., Bierling, P., Hernigou, P., Layrolle, P., Rouard, H., 2010. Osteoblastic differentiation of human mesenchymal stem cells with platelet lysate. Biomaterials 31 2 , 270–278. doi:10.1016/j.biomaterials.2009.09.043
28. Cho, C.-B., Jung, S.Y., Park, C.Y., Kang, H.K., Yeo, I.-S.L., Min, B.-M., 2019. A Vitronectin-Derived Bioactive Peptide Improves Bone Healing Capacity of SLA Titanium Surfaces. Materials (Basel) 12 20 . doi:10.3390/ma12203400
29. Chou, W.-C., Wang, R.C.-C., Liu, C., Yang, C.-Y., Lee, T.-M., 2017. Surface Modification of Direct-Current and Radio-Frequency Oxygen Plasma Treatments Enhance Cell Biocompatibility. Materials (Basel) 10 11 . doi:10.3390/ma10111223
30. Chrcanovic, B.R., Kisch, J., Albrektsson, T., Wennerberg, A., 2016. Factors Influencing Early Dental Implant Failures. J. Dent. Res. 95 9 , 995–1002. doi:10.1177/0022034516646098
31. Classification of Bones | SEER Training n.d. URL https://training.seer.cancer.gov/anatomy/skeletal/classification.html
32. Coathup, M.J., Blunn, G.W., Mirhosseini, N., Erskine, K., Liu, Z., Garrod, D.R., Li, L., 2017. Controlled laser texturing of titanium results in reliable osteointegration: OSTEOINTEGRATION OF LASER TEXTURED IMPLANTS. J. Orthop. Res. 35 4 , 820–828. doi:10.1002/jor.23340
33. Cohen, M.M., 2000. Merging the old skeletal biology with the new. I. Intramembranous ossification, endochondral ossification, ectopic bone, secondary cartilage, and pathologic considerations. J. Craniofac. Genet. Dev. Biol. 20 2 , 84–93.
34. Comparison of bone morphogenetic protein-2 delivery systems to induce supracrestal bone guided by titanium implants in the rabbit mandible - Wen - 2016 - Clinical Oral Implants Research - Wiley Online Library n.d. URL https://onlinelibrary.wiley.com/doi/abs/10.1111/clr.12645
35. del Cerro, M., Cogen, J., del Cerro, C., 1980. Stevenel's Blue, an excellent stain for optical microscopical study of plastic embedded tissues. Microsc Acta 83 2 , 117–121.
36. Delany, A.M., Hankenson, K.D., 2009. Thrombospondin-2 and SPARC/osteonectin are critical regulators of bone remodeling. J. Cell Commun. Signal. 3 3–4 , 227–238. doi:10.1007/s12079-009-0076-0
37. Deschaseaux, F., Sensébé, L., Heymann, D., 2009. Mechanisms of bone repair and regeneration. Trends in Molecular Medicine 15 9 , 417–429. doi:10.1016/j.molmed.2009.07.002

38. Doe, Y., Ida, H., Seiryu, M., Deguchi, T., Takeshita, N., Sasaki, Satoshi, Sasaki, Shutaro, Irie, D., Tsuru, K., Ishikawa, K., Takano-Yamamoto, T., 2020. Titanium surface treatment by calcium modification with acid-etching promotes osteogenic activity and stability of dental implants. Materialia 12, 100801. doi:10.1016/j.mtla.2020.100801
39. Dole, N.S., Mazur, C.M., Acevedo, C., Lopez, J.P., Monteiro, D.A., Fowler, T.W., Gludovatz, B., Walsh, F., Regan, J.N., Messina, S., Evans, D.S., Lang, T.F., Zhang, B., Ritchie, R.O., Mohammad, K.S., Alliston, T., 2017. Osteocyte-Intrinsic TGF-β Signaling Regulates Bone Quality through Perilacunar/Canalicular Remodeling. Cell Reports 21 9 , 2585–2596. doi:10.1016/j.celrep.2017.10.115
40. During, A., Penel, G., Hardouin, P., 2015. Understanding the local actions of lipids in bone physiology. Progress in Lipid Research 59, 126–146. doi:10.1016/j.plipres.2015.06.002
41. Ehnert, S., Baur, J., Schmitt, A., Neumaier, M., Lucke, M., Dooley, S., Vester, H., Wildemann, B., Stöckle, U., Nussler, A.K., 2010. TGF-β1 As Possible Link between Loss of Bone Mineral Density and Chronic Inflammation. PLOS ONE 5 11 , e14073. doi:10.1371/journal.pone.0014073
42. Emans, P.J., Spaapen, F., Surtel, D.A.M., Reilly, K.M., Cremers, A., van Rhijn, L.W., Bulstra, S.K., Voncken, J.W., Kuijer, R., 2007. A novel in vivo model to study endochondral bone formation; HIF-1alpha activation and BMP expression. Bone 40 2 , 409–418. doi:10.1016/j.bone.2006.08.005
43. Farrell, K.B., Karpeisky, A., Thamm, D.H., Zinnen, S., 2018. Bisphosphonate conjugation for bone specific drug targeting. Bone Reports 9, 47–60. doi:10.1016/j.bonr.2018.06.007
44. Ferraris, S., Spriano, S., Bianchi, C.L., Cassinelli, C., Vernè, E., 2011. Surface modification of Ti-6Al-4 V alloy for biomineralization and specific biological response: part II, alkaline phosphatase grafting. J Mater Sci Mater Med 22 8 , 1835–1842. doi:10.1007/s10856-011-4365-9
45. Filipowska, J., Tomaszewski, K.A., Niedźwiedzki, Ł., Walocha, J.A., Niedźwiedzki, T., 2017b. The role of vasculature in bone development, regeneration and proper systemic functioning. Angiogenesis 20 3 , 291–302. doi:10.1007/s10456-017-9541-1
46. Foster, B.L., Ao, M., Salmon, C.R., Chavez, M.B., Kolli, T.N., Tran, A.B., Chu, E.Y., Kantovitz, K.R., Yadav, M., Narisawa, S., Millán, J.L., Nociti, F.H., Somerman, M.J., 2018. Osteopontin regulates dentin and alveolar bone development and mineralization. Bone 107, 196–207. doi:10.1016/j.bone.2017.12.004
47. Fracture Healing - Basic Science - Orthobullets n.d. URL https://www.orthobullets.com/basic-science/9009/fracture-healing
48. González-Blanco, C., Rizo-Gorrita, M., Luna-Oliva, I., Serrera-Figallo, M.-Á., Torres-Lagares, D., Gutiérrez-Pérez, J.-L., 2019. Human Osteoblast Cell Behaviour on Titanium Discs Treated with Argon Plasma. Materials (Basel) 12 11 . doi:10.3390/ma12111735
49. Granato, R., Bonfante, E.A., Castellano, A., Khan, R., Jimbo, R., Marin, C., Morsi, S., Witek, L., Coelho, P.G., 2019. Osteointegrative and

microgeometric comparison between micro-blasted and alumina blasting/acid etching on grade II and V titanium alloys (Ti-6Al-4V). Journal of the Mechanical Behavior of Biomedical Materials 97, 288–295. doi:10.1016/j.jmbbm.2019.05.026

50. Grimaud, E., Heymann, D., Rédini, F., 2002. Recent advances in TGF-β effects on chondrocyte metabolism: Potential therapeutic roles of TGF-β in cartilage disorders. Cytokine & Growth Factor Reviews 13 3 , 241–257. doi:10.1016/S1359-6101(02)00004-7
51. Guang, M., Huang, B., Yao, Y., Zhang, L., Yang, B., Gong, P., 2017. Effects of vascular endothelial growth factor on osteoblasts around dental implants in vitro and in vivo. J Oral Sci 59 2 , 215–223. doi:10.2334/josnusd.16-0406
52. Hall, B., Miyake, T., 2000. All for one and one for all: Condensations and the initiation of skeletal development. BioEssays 22, 138–147. doi:10.1002/(SICI)1521-1878(200002)22:2<138::AID-BIES5>3.0.CO;2-4
53. Han, J., Hong, G., Lin, H., Shimizu, Y., Wu, Y., Zheng, G., Zhang, H., Sasaki, K., 2016. Biomechanical and histological evaluation of the osseointegration capacity of two types of zirconia implant. Int J Nanomedicine 11, 6507–6516. doi:10.2147/IJN.S119519
54. Hankenson, K.D., Dishowitz, M., Gray, C., Schenker, M., 2011. Angiogenesis in Bone Regeneration. Injury 42 6 , 556–561. doi:10.1016/j.injury.2011.03.035
55. Hayakawa, T., Kiba, H., Yasuda, S., Yamamoto, H., Nemoto, K., 2002. A histologic and histomorphometric evaluation of two types of retrieved human titanium implants. Int J Periodontics Restorative Dent 22 2 , 164–171.
56. Hinoi, E., Bialek, P., Chen, Y.-T., Rached, M.-T., Groner, Y., Behringer, R.R., Ornitz, D.M., Karsenty, G., 2006. Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. Genes Dev. 20 21 , 2937–2942. doi:10.1101/gad.1482906
57. Izquierdo-Barba, I., Santos-Ruiz, L., Becerra, J., Feito, M.J., Fernández-Villa, D., Serrano, M.C., Díaz-Güemes, I., Fernández-Tomé, B., Enciso, S., Sánchez-Margallo, F.M., Monopoli, D., Afonso, H., Portolés, M.T., Arcos, D., Vallet-Regí, M., 2019. Synergistic effect of Si-hydroxyapatite coating and VEGF adsorption on Ti6Al4V-ELI scaffolds for bone regeneration in an osteoporotic bone environment. Acta Biomaterialia 83, 456–466. doi:10.1016/j.actbio.2018.11.017
58. Jahangir, A.A., Nunley, R.M., Mehta, S., Sharan, A., n.d. Bone-graft substitutes in orthopaedic surgery 5.
59. Jang, T.-H., Park, J.-H., Moon, W., Chae, J.-M., Chang, N.-Y., Kang, K.-H., 2018. Effects of acid etching and calcium chloride immersion on removal torque and bone-cutting ability of orthodontic mini-implants. American Journal of Orthodontics and Dentofacial Orthopedics 154 1 , 108–114. doi:10.1016/j.ajodo.2017.10.032
60. Janssens, K., ten Dijke, P., Janssens, S., Van Hul, W., 2005. Transforming Growth Factor-β1 to the Bone. Endocr Rev 26 6 , 743–774. doi:10.1210/er.2004-0001
61. Jayesh, R.S., Dhinakarsamy, V., 2015. Osseointegration. J Pharm Bioallied Sci 7 Suppl 1 , S226–S229. doi:10.4103/0975-7406.155917

62. Jemat, A., Ghazali, M.J., Razali, M., Otsuka, Y., 2015. Surface Modifications and Their Effects on Titanium Dental Implants BioMed Research International. doi:https://doi.org/10.1155/2015/791725
63. Katagiri, T., Tsukamoto, S., Nakachi, Y., Kuratani, M., 2018. Discovery of Heterotopic Bone-Inducing Activity in Hard Tissues and the TGF-β Superfamily. Int J Mol Sci 19 11 . doi:10.3390/ijms19113586
64. Khojasteh, A., Behnia, H., Naghdi, N., Esmaeelinejad, M., Alikhassy, Z., Stevens, M., 2013. Effects of different growth factors and carriers on bone regeneration: a systematic review. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology 116 6 , e405–e423. doi:10.1016/j.oooo.2012.01.044
65. Kim, H., Choi, S.-H., Ryu, J.-J., Koh, S.-Y., Park, J.-H., Lee, I.-S., 2008. The biocompatibility of SLA-treated titanium implants. Biomed. Mater. 3 2 , 025011. doi:10.1088/1748-6041/3/2/025011
66. Kim, S.K., Lee, J.B., Koak, J.Y., Heo, S.J., Lee, K.R., Cho, L.R., Lee, S.S., 2005. An abutment screw loosening study of a Diamond Like Carbon-coated CP titanium implant. Journal of Oral Rehabilitation 32 5 , 346–350. doi:10.1111/j.1365-2842.2004.01475.x
67. Knight, M.N., Hankenson, K.D., 2013. Mesenchymal Stem Cells in Bone Regeneration. Adv Wound Care (New Rochelle) 2 6 , 306–316. doi:10.1089/wound.2012.0420
68. Kokubo, T., Kim, H.-M., Kawashita, M., 2003. Novel bioactive materials with different mechanical properties. Biomaterials, Focus on Biomaterials Science in Asia 24 13 , 2161–2175. doi:10.1016/S0142-9612(03)00044-9
69. Komori, T., 2019. Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. International Journal of Molecular Sciences 20 7 , 1694. doi:10.3390/ijms20071694
70. Lang, N.P., Salvi, G.E., Huynh-Ba, G., Ivanovski, S., Donos, N., Bosshardt, D.D., 2011. Early osseointegration to hydrophilic and hydrophobic implant surfaces in humans: Early osseointegration on implant surfaces. Clinical Oral Implants Research 22 4 , 349–356. doi:10.1111/j.1600-0501.2011.02172.x
71. Le Guéhennec, L., Soueidan, A., Layrolle, P., Amouriq, Y., 2007. Surface treatments of titanium dental implants for rapid osseointegration. Dental Materials 23 7 , 844–854. doi:10.1016/j.dental.2006.06.025
72. Lee, J.-B., Jo, Y.-H., Choi, J.-Y., Seol, Y.-J., Lee, Y.-M., Ku, Y., Rhyu, I.-C., Yeo, I.-S.L., 2019. The Effect of Ultraviolet Photofunctionalization on a Titanium Dental Implant with Machined Surface: An In Vitro and In Vivo Study. Materials (Basel) 12 13 . doi:10.3390/ma12132078
73. Lindahl, A., Brittberg, M., Gibbs, D., Dawson, J.I., Kanczler, J., Black, C., Tare, R., Oreffo, R.O.C., 2015. Chapter 16 - Cartilage and Bone Regeneration, in: Blitterswijk, C.A.V., De Boer, J. (Eds.), Tissue Engineering (Second Edition). Academic Press, Oxford, pp. 529–582. doi:10.1016/B978-0-12-420145-3.00016-X
74. Liu, X., Chu, P.K., Ding, C., 2004. Surface modification of titanium, titanium alloys, and related materials for biomedical applications. Materials Science & Engineering, R 47 3–4 , 49–121. doi:10.1016/j.mser.2004.11.001

75. Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25 4 , 402–408. doi:10.1006/meth.2001.1262
76. Lotz, E.M., Berger, M.B., Schwartz, Z., Boyan, B.D., 2018. Regulation of Osteoclasts by Osteoblast Lineage Cells Depends on Titanium Implant Surface Properties. Acta Biomater 68, 296–307. doi:10.1016/j.actbio.2017.12.039
77. Ma, T., Ge, X.-Y., Hao, K.-Y., Zhang, B.-R., Jiang, X., Lin, Y., Zhang, Y., 2017. Simple 3,4-Dihydroxy-L-Phenylalanine Surface Modification Enhances Titanium Implant Osseointegration in Ovariectomized Rats. Sci Rep 7. doi:10.1038/s41598-017-18173-5
78. Maes, C., Carmeliet, G., 2013. Vascular and Nonvascular Roles of VEGF in Bone Development, Madame Curie Bioscience Database [Internet]. Landes Bioscience.
79. Mak, K.K., Bi, Y., Wan, C., Chuang, P.-T., Clemens, T., Young, M., Yang, Y., 2008. Hedgehog signaling in mature osteoblasts regulates bone formation and resorption by controlling PTHrP and RANKL expression. Dev. Cell 14 5 , 674–688. doi:10.1016/j.devcel.2008.02.003
80. Mändl, S., Sader, R., Thorwarth, G., Krause, D., Zeilhofer, H.-F., Horch, H.H., Rauschenbach, B., 2002. Investigation on plasma immersion ion implantation treated medical implants. Biomol. Eng. 19 2–6 , 129–132. doi:10.1016/s1389-0344(02)00025-4
81. Maniscalco, W.M., D'Angio, C.T., 2006. VASCULAR ENDOTHELIAL GROWTH FACTOR, in: Laurent, G.J., Shapiro, S.D. (Eds.), Encyclopedia of Respiratory Medicine. Academic Press, Oxford, pp. 413–418. doi:10.1016/B0-12-370879-6/00434-8
82. Martins, R., Cestari, T.M., Arantes, R.V.N., Santos, P.S., Taga, R., Carbonari, M.J., Oliveira, R.C., 2018. Osseointegration of zirconia and titanium implants in a rabbit tibiae model evaluated by microtomography, histomorphometry and fluorochrome labeling analyses. Journal of Periodontal Research 53 2 , 210–221. doi:10.1111/jre.12508
83. Mei, S., Dong, F., Rahman Khan, M.S., 2018. Effects of Biomineralization on Osseointegration of Pure Titanium Implants in the Mandible of Beagles. Journal of Oral and Maxillofacial Surgery 76 10 , 2104.e1-2104.e10. doi:10.1016/j.joms.2018.06.015
84. Meskinfam, M., 2017. 17 - Polymer scaffolds for bone regeneration, in: Tanzi, M.C., Farè, S. (Eds.), Characterization of Polymeric Biomaterials. Woodhead Publishing, pp. 441–475. doi:10.1016/B978-0-08-100737-2.00017-0
85. Minami, M., Takechi, M., Ohta, K., Ohta, A., Ninomiya, Y., Takamoto, M., Fukui, A., Tada, M., Kamata, N., 2013. Bone formation and osseointegration with titanium implant using granular- and block-type porous hydroxyapatite ceramics (IP-CHA). Dent Mater J 32 5 , 753–760. doi:10.4012/dmj.2012-169
86. Minardi, S., Taraballi, F., Cabrera, F.J., Van Eps, J., Wang, X., Gazze, S.A., Fernandez-Mourev, J.S., Tampieri, A., Francis, L., Weiner, B.K., Tasciotti, E., 2019. Biomimetic hydroxyapatite/collagen composite drives bone niche

recapitulation in a rabbit orthotopic model. Mater Today Bio 2. doi:10.1016/j.mtbio.2019.100005
87. Minkin, C., Marinho, V.C., 1999. Role of the Osteoclast at the Bone-Implant Interface. Adv Dent Res. 13 1 , 49–56. doi:10.1177/08959374990130011401
88. Morgan, E.F., Unnikrisnan, G.U., Hussein, A.I., 2018. Bone Mechanical Properties in Healthy and Diseased States. Annu Rev Biomed Eng 20, 119–143. doi:10.1146/annurev-bioeng-062117-121139
89. Na, Y., Heo, S.-J., Kim, S.-K., Koak, J.-Y., 2009. Implant surface treatments affect gene expression of Runx2, osteogenic key marker. J Adv Prosthodont 1 2 , 91–96. doi:10.4047/jap.2009.1.2.91
90. Najeeb, S., Zafar, M.S., Khurshid, Z., Zohaib, S., Hasan, S.M., Khan, R.S., 2017. Bisphosphonate releasing dental implant surface coatings and osseointegration: A systematic review. Journal of Taibah University Medical Sciences 12 5 , 369–375. doi:10.1016/j.jtumed.2017.05.007
91. Nakamura, Y., Wang, X., Xu, C., Asakura, A., Yoshiyama, M., From, A.H.L., Zhang, J., 2007. Xenotransplantation of Long-Term-Cultured Swine Bone Marrow-Derived Mesenchymal Stem Cells. STEM CELLS 25 3 , 612–620. doi:10.1634/stemcells.2006-0168
92. Nautiyal, V.P., Mittal, A., Agarwal, A., Pandey, A., 2013. Tissue response to titanium implant using scanning electron microscope. Natl J Maxillofac Surg 4 1 , 7–12. doi:10.4103/0975-5950.117815
93. Neman, J., Hambrecht, A., Cadry, C., Jandial, R., 2012. Stem cell-mediated osteogenesis: therapeutic potential for bone tissue engineering. Biologics 6, 47–57. doi:10.2147/BTT.S22407
94. Noda, M., Denhardt, D.T., 2008. Chapter 18 - Osteopontin, in: Bilezikian, J.P., Raisz, L.G., Martin, T.J. (Eds.), Principles of Bone Biology (Third Edition). Academic Press, San Diego, pp. 351–366. doi:10.1016/B978-0-12-373884-4.00037-9
95. Noonan, K.J., Hunziker, E.B., Nessler, J., Buckwalter, J.A., 1998. Changes in cell, matrix compartment, and fibrillar collagen volumes between growth-plate zones. J. Orthop. Res. 16 4 , 500–508. doi:10.1002/jor.1100160416
96. Novaes Jr, A.B., Souza, S.L.S. de, Barros, R.R.M. de, Pereira, K.K.Y., Iezzi, G., Piattelli, A., 2010. Influence of implant surfaces on osseointegration. Braz. Dent. J. 21 6 , 471–481. doi:10.1590/S0103-64402010000600001
97. Oryan, A., Alidadi, S., Moshiri, A., 2013. Current concerns regarding healing of bone defects. Hard Tissue 2 2 . doi:10.13172/2050-2303-2-2-374
98. Osseointegration of bone implants: A review of an alternative mode of fixation: Acta Orthopaedica Scandinavica: Vol 58, No 5 n.d. URL https://www.tandfonline.com/doi/abs/10.3109/17453678709146401
99. Osteoblast response to nanostructured and phosphorus-enhanced titanium anodization surfaces - Sakshi Jain, R Scott Williamson, Amol V Janorkar, Jason A Griggs, Michael D Roach, 2019], n.d. URL https://journals.sagepub.com/doi/abs/10.1177/0885328219852741
100. Osteonectin, 2020. . Wikipedia.
101. Owen, R., Reilly, G.C., 2018. In vitro Models of Bone Remodelling and Associated Disorders. Front. Bioeng. Biotechnol. 6. doi:10.3389/fbioe.2018.00134

102. Pang, X., Gong, K., Zhang, X., Wu, S., Cui, Y., Qian, B.-Z., 2019. Osteopontin as a multifaceted driver of bone metastasis and drug resistance. Pharmacological Research 144, 235–244. doi:10.1016/j.phrs.2019.04.030
103. Parvizi, J., Kim, G.K., 2010. Chapter 163 - Osteoclasts, in: Parvizi, J., Kim, G.K. (Eds.), High Yield Orthopaedics. W.B. Saunders, Philadelphia, pp. 337–339. doi:10.1016/B978-1-4160-0236-9.00174-7
104. Pontes, A.E.F., de Toledo, C.T., Garcia, V.G., Ribeiro, F.S., Sakakura, C.E., 2015. Torque Analysis of a Triple Acid-Etched Titanium Implant Surface [WWW Document]. The Scientific World Journal. doi:https://doi.org/10.1155/2015/819879
105. Qiu, Z.-Y., Cui, Y., Wang, X.-M., 2019. Natural Bone Tissue and Its Biomimetic, in: Mineralized Collagen Bone Graft Substitutes. Elsevier, pp. 1–22. doi:10.1016/B978-0-08-102717-2.00001-1
106. Raines, A.L., Berger, M.B., Patel, N., Hyzy, S.L., Boyan, B.D., Schwartz, Z., 2019. VEGF-A regulates angiogenesis during osseointegration of Ti implants via paracrine/autocrine regulation of osteoblast response to hierarchical microstructure of the surface. J Biomed Mater Res A 107 2 , 423–433. doi:10.1002/jbm.a.36559
107. Raphel, J., Karlsson, J., Galli, S., Wennerberg, A., Lindsay, C., Haugh, M., Pajarinen, J., Goodman, S.B., Jimbo, R., Andersson, M., Heilshorn, S.C., 2016. Engineered Protein Coatings to Improve the Osseointegration of Dental and Orthopaedic Implants. Biomaterials 83, 269–282. doi:10.1016/j.biomaterials.2015.12.030
108. Rauch, F., 2014b. Bone Histomorphometry, in: Osteogenesis Imperfecta. Elsevier, pp. 237–242. doi:10.1016/B978-0-12-397165-4.00025-3
109. Romero-Ruiz, M.M., Gil-Mur, F.J., Ríos-Santos, J.V., Lázaro-Calvo, P., Ríos-Carrasco, B., Herrero-Climent, M., 2019. Influence of a Novel Surface of Bioactive Implants on Osseointegration: A Comparative and Histomorfometric Correlation and Implant Stability Study in Minipigs. Int J Mol Sci 20 9 . doi:10.3390/ijms20092307
110. Rosset, E.M., Bradshaw, A.D., 2016. SPARC/Osteonectin in Mineralized Tissue. Matrix Biol 52–54, 78–87. doi:10.1016/j.matbio.2016.02.001
111. Rosset, E.M., Bradshaw, A.D., 2016. SPARC/Osteonectin in Mineralized Tissue. Matrix Biol 52–54, 78–87. doi:10.1016/j.matbio.2016.02.001

112. Sawai, A.A.A., Rajmohan, C.S., Labib, H., Tabiuk, S., 2017. Advances in Dental Implant Positioning Techniques and Their Clinical Implications. OJST 07 02 , 121–135. doi:10.4236/ojst.2017.72008
113. Shan, L., Kadhum, A.A.H., Al-Furjan, M.S.H., Weng, W., Gong, Y., Cheng, K., Zhou, M., Dong, L., Chen, G., Takriff, M.S., Sulong, A.B., 2019. In Situ Controlled Surface Microstructure of 3D Printed Ti Alloy to Promote Its Osteointegration. Materials (Basel) 12 5 . doi:10.3390/ma12050815

114. Shrivats, A.R., Alvarez, P., Schutte, L., Hollinger, J.O., 2014. Bone Regeneration, in: Principles of Tissue Engineering. Elsevier, pp. 1201–1221. doi:10.1016/B978-0-12-398358-9.00055-0
115. Singh, A., Gill, G., Kaur, H., Amhmed, M., Jakhu, H., 2018. Role of osteopontin in bone remodeling and orthodontic tooth movement: a review. Prog Orthod 19. doi:10.1186/s40510-018-0216-2
116. Sivaraman, K., Chopra, A., Narayan, A.I., Balakrishnan, D., 2018. Is zirconia a viable alternative to titanium for oral implant? A critical review. Journal of Prosthodontic Research 62 2 , 121–133. doi:10.1016/j.jpor.2017.07.003
117. Smeets, R., Stadlinger, B., Schwarz, F., Beck-Broichsitter, B., Jung, O., Precht, C., Kloss, F., Gröbe, A., Heiland, M., Ebker, T., 2016. Impact of Dental Implant Surface Modifications on Osseointegration. BioMed Research International 2016, 1–16. doi:10.1155/2016/6285620
118. Starosvetsky, D., Gotman, I., 2001. Corrosion behavior of titanium nitride coated Ni–Ti shape memory surgical alloy. Biomaterials 22 13 , 1853–1859. doi:10.1016/S0142-9612(00)00368-9
119. Stegen, S., van Gastel, N., Carmeliet, G., 2015. Bringing new life to damaged bone: The importance of angiogenesis in bone repair and regeneration. Bone, Stem Cells and Bone 70, 19–27. doi:10.1016/j.bone.2014.09.017
120. Stewart, S., Bryant, S.J., Ahn, J., Hankenson, K.D., 2015. Chapter 24 - Bone Regeneration, in: Atala, A., Allickson, J.G. (Eds.), Translational Regenerative Medicine. Academic Press, Boston, pp. 313–333. doi:10.1016/B978-0-12-410396-2.00024-4
121. Surface modification of Ti-6Al-4 v alloy for biomineralization and specific biological response: Part II, alkaline phosphatase grafting | n.d. URL https://www.researchgate.net/publication/51208010_Surface_modification_of_Ti-6Al-4_v_alloy_for_biomineralization_and_specific_biological_response_Part_II_alkaline_phosphatase_grafting
122. Surface Modifications and Their Effects on Titanium Dental Implants. - n.d. . docksci.com. URL https://docksci.com/surface-modifications-and-their-effects-on-titanium-dental-implants_5a2bc1c7d64ab2354651de9a.html
123. Surface Treatments of Titanium Dental Implants for Rapid Osseointegration - n.d. URL https://pubmed.ncbi.nlm.nih.gov/16904738/
124. Suzuki, T., Hori, N., Att, W., Kubo, K., Iwasa, F., Ueno, T., Maeda, H., Ogawa, T., 2009. Ultraviolet Treatment Overcomes Time-Related Degrading Bioactivity of Titanium. Tissue Engineering Part A 15 12 , 3679–3688. doi:10.1089/ten.tea.2008.0568
125. Thielen, N.G.M., van der Kraan, P.M., van Caam, A.P.M., 2019. TGFβ/BMP Signaling Pathway in Cartilage Homeostasis. Cells 8 9 . doi:10.3390/cells8090969
126. Tibau, A.V., Grube, B.D., Velez, B.J., Vega, V.M., Mutter, J., 2019. Titanium exposure and human health. Oral Sci Int 16 1 , 15–24. doi:10.1002/osi2.1001

127. Ting, M., Jefferies, S.R., Xia, W., Engqvist, H., Suzuki, J.B., 2017. Classification and Effects of Implant Surface Modification on the Bone: Human Cell–Based In Vitro Studies. J Oral Implantol 43 1 , 58–83. doi:10.1563/aaid-joi-D-16-00079
128. TY - JOURAU - Hall, BrianAU - Miyake, TsutomuPY - 2000/01/31SP - 138 EP - 147T1 - All for one and one for all: Condensat, n.d.
129. Uskoković, V., Desai, T.A., 2013. Phase composition control of calcium phosphate nanoparticles for tunable drug delivery kinetics and treatment of osteomyelitis. II. Antibacterial and osteoblastic response. J Biomed Mater Res A 101 5 , 1427–1436. doi:10.1002/jbm.a.34437
130. Velasco-Ortega, E., Ortiz-García, I., Jiménez-Guerra, A., Monsalve-Guil, L., Muñoz-Guzón, F., Perez, R.A., Gil, F.J., 2019. Comparison between Sandblasted Acid-Etched and Oxidized Titanium Dental Implants: In Vivo Study. Int J Mol Sci 20 13 . doi:10.3390/ijms20133267
131. Wachesk, C.C., Trava-Airoldi, V.J., Da-Silva, N.S., Lobo, A.O., Marciano, F.R., 2016. The Influence of Titanium Dioxide on Diamond-Like Carbon Biocompatibility for Dental Applications Journal of Nanomaterials. doi:https://doi.org/10.1155/2016/8194516
132. Wachira, E., Tran, K., Taylor, S., Hoger, S., Dunn, J., 2016. Genotyping and Resolution of a Case of Osteomyelitis in a 16-Month-Old Boy of Hispanic/African American Ethnicity. Lab Med 47 1 , 57–62. doi:10.1093/labmed/lmv001
133. Wennerberg, A., Albrektsson, T., 2009. Effects of titanium surface topography on bone integration: a systematic review. Clin Oral Implants Res 20 Suppl 4, 172–184. doi:10.1111/j.1600-0501.2009.01775.x
134. Wong, J.Y., Bronzino, J.D., Peterson, D.R., 2012. Biomaterials: Principles and Practices. CRC Press.
135. Wu, M., Chen, G., Li, Y.-P., 2016. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res 4, 16009. doi:10.1038/boneres.2016.9
136. Xenotransplantation of Long-Term-Cultured Swine Bone Marrow-Derived Mesenchymal Stem Cells - Nakamura - 2007 - STEM CELLS - Wiley Online Library n.d. URL https://stemcellsjournals.onlinelibrary.wiley.com/doi/full/10.1634/stemcells.2006-0168
137. Yadav, A., Yadav, R., Gupta, A., Baranwal, A., Bhatnagar, A., Singh, V., 2017. Effect of Ultraviolet Irradiation on the Osseointegration of a Titanium Alloy with Bone. Contemp Clin Dent 8 4 , 571–578. doi:10.4103/ccd.ccd_576_17
138. Yaszemski, M.J., Payne, R.G., Hayes, W.C., Langer, R., Mikos, A.G., 1996. Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. Biomaterials, Polymer Scaffolding and Hard Tissue Engineering 17 2 , 175–185. doi:10.1016/0142-9612(96)85762-0
139. Zhang, H., Aronow, M.S., Gronowicz, G.A., 2005. Transforming growth factor-beta 1 (TGF-β1) prevents the age-dependent decrease in bone formation in human osteoblast/implant cultures. Journal of Biomedical Materials Research Part A 75A 1 , 98–105. doi:10.1002/jbm.a.30400

140. Zhao, X., Ren, X., Wang, C., Huang, B., Ma, J., Ge, B., Jia, Z., Li, Y., 2020. Enhancement of hydroxyapatite formation on titanium surface by alkali heat treatment combined with induction heating and acid etching. Surface and Coatings Technology 399, 126173. doi:10.1016/j.surfcoat.2020.126173

141. Zhao, Z., Zhao, M., Xiao, G., Franceschi, R.T., 2005. Gene Transfer of the Runx2 Transcription Factor Enhances Osteogenic Activity of Bone Marrow Stromal Cells in Vitro and in Vivo. Molecular Therapy 12 2 , 247–253. doi:10.1016/j.ymthe.2005.03.009

142. Zhu, X., Chen, J., Scheideler, L., Reichl, R., Geis-Gerstorfer, J., 2004a. Effects of topography and composition of titanium surface oxides on osteoblast responses. Biomaterials 25 18 , 4087–4103. doi:10.1016/j.biomaterials.2003.11.011