Long Term Osseointegration Study of Laser Additive Manufactured Commercially Pure Titanium (LAM-Cp-Ti) implants in Rabbit Femur Model by Histopathological and Molecular Analysis

A DISSERTATION SUBMITTED BY

GAYATHRY G

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DECLARATION

I, **Gayathry G**, hereby declare that I had personally carried out the work depicted in the thesis entitled, **"Long term Osseointegration study of Laser Additive Manufactured Commercially Pure Titanium (LAM-Cp-Ti) Implants in Rabbit femur model by Histopathological and Molecular Analysis"**under the direct supervision of **Dr.Sabareeswaran A,Scientist E,Histopathology lab**, Division of Experimental Pathology, Department of Applied Biology, BiomedicalTechnology Wing, SreeChitraTirunalInstitute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Thiruvananthapuram GayathryG

Date: 2017/MPhil/03

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES

AND TECHNOLOGY

THIRUVANANTHAPURAM – 695011, INDIA

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CERTIFICATE

This is to certify that the dissertation entitled**"Long term Osseointegration study of Laser Additive Manufactured Commercially Pure Titanium (LAM-Cp-Ti) Implants in Rabbit Femur Model by Histopathological and Molecular Analysis"** is a bonafide work done by **Ms.Gayathry G**in partial fulfilment for the degree of **Master in Philosophy** under mysupervision and guidance at **Histopathology lab**, BiomedicalTechnology Wing, SreeChitraTirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Thiruvananthapuram Dr.SabareeswaranA

Date: Scientist E, Histopathology

The Thesis entitled

Long term Osseointegration study of Laser Additive Manufactured Commercially Pure Titanium (LAM-Cp-Ti) Implants in Rabbit Femur Model by Histopathological and Molecular Analysis

Submitted by

Gayathry G

For the degree of

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From

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

Thiruvananthapuram – 695011, INDIA

Evaluated and approved by

Signature **Signature**

Name of supervisor **Examiner's name and designation**

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ABBREVIATIONS

3DP	3 Dimensional printing
AM	Additive manufacturing
ALP	Alkaline phosphatase
ASTM	American Society for Testing and Materials
BMU	Basic Multicellular Unit
BMPs	Bone morphogenetic proteins
Cb	Cortical bone
CO2	Carbon dioxide
Co-Cr	Cobalt-Chromium
COL 1	Collagen 1
Cp-Ti	Commercially pure titanium
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
ECM	Extracellular matrix
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FDM	Fused deposition modeling

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
I	Implant
IGF 1	Insulin growth factor 1
IL 6	Interleukin 6
ISO	International Organization for Standardization
LOM	Laminated Object Manufacturing
LAM-Cp-Ti	Laser additive manufactured commercially pure titanium
LM	Laser Melting
LMD	Laser metal deposition
LS	Laser sintering
M	Matrix
MSCs	Mesenchymal stem cells
MMA	Methyl methacrylate
NIH	National Institutes of health
OBL	Osteoblast
OD	Optical density
Ob	Old bone

OPG	Osteoprotogenin
PTH	Parathyroid hormone
PMMA	Polymethyl methacrylate
RT-PCR	Real-time polymerase chain reaction
PMNs	Polymorphonuclear neutrophils
RRCAT	Raja Ramanna Centre for Advanced Technology
RANKL	Receptor Activator of Nuclear κB Ligand
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
SLS	Selective Laser Sintering
SPARC	Secreted protein acidic and rich in cysteine
SS	Stainless steel
SD	Standard deviation
SLA	Stereolithography
Ti	Titanium

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SYNOPSIS

Titanium is found to be a better biomaterial, which stands out due to its excellent physiochemical properties when compared to other metallic biomaterials (e.g. stainless steel and cobalt chromium alloys). Both the commercially pure titanium (Cp-Ti) and its alloys show better corrosion resistance than any other material when contacted with human bone, body fluids, and soft tissue. Titanium materials are usually fabricated by conventional methods such as milling, casting, and powder metallurgy and involve considerable material and energy expenditure and make the fabrication processes tedious and time intensive. In addition, orthopedic implants are required to conform to the complex geometry of human bones and joints which makes their fabrication difficult. Additive manufacturing has been widely used for the fabrication of various metallic materials. Laser Additive Manufacturing is one of the advanced additive manufacturing processes that are capable of fabricating engineering components directly from a solid model. As a result, the fabricated structure is highly customized and expected to have biomechanical properties, which are comparable to autogenous tissues without adverse effects. Research now centers to improve the osseointegration capability of titanium implants by morphological and chemical surface modification.

Chapter 1 introduces the background of the study, reviews the literature on the previous studies conducted and has cited the main hypothesis and objectives of the present study. Literature review concentrates on bone, cellular components of bone, bone remodeling signaling molecules that accelerate bone formation by acting on transcriptional level, bone fracture, different implants used, tissue response to implantation, events at bone-

implant interface, titanium and its biomedical applications, laser additive manufactured titanium implants, animal model used for the study.

Chapter 2 describes the methods and the materials used in conducting the histologic and osteogenic gene expression study. Approval has been obtained from Institutional Animal Ethics Committee. Animal implantation studies were conducted in five New Zealand White rabbit as per ISO 10993-6 standard. In left femur bone, Laser manufactured commercially pure titanium (LAM-Cp-Ti) was implanted and in right femur, Cp-Ti was implanted. Animals were sacrificed post six months of implantation. For histology, bone tissue with the implant was harvested, fixed in 10% neutral buffer formalin and embedded in polymeric resin. Sections were cut using high precision saw microtome, ground and polished. Sections were stained using Stevenel's blue and Van Gieson picrofuchsin stain and evaluated microscopically. For molecular studies, bone samples were crushed in liquid nitrogen and stored in the trizol reagent. Ribose nucleic acid (RNA) was isolated following guanidium-phenol method for osteogenesis associated gene expression studies. Osteogenic genes namely RUNX2, SPARC (Osteonectin) and Collagen 1 were analyzed using Real Time Polymerase chain reaction (RT PCR).

Chapter 3 details the results of animal experiments, gross and histological features and osteogenic gene expression. Qualitative analysis was done using histological staining methods which revealed the presence of new bone and active osteoblast cells indicating new bone formation and bone remodeling. Three types of comparison were made to analyze the expression of RUNX2, SPARC and Collagen 1 genes in the newly formed bone at the bone -implant interface. In the newly formed bone around LAM-Cp-Ti implant, when compared with normal cortical bone, upregulation of Collagen 1 and SPARC genes and down regulation of RUNX2 gene was observed in LAM-Cp-Ti. In the newly formed bone around Cp-Ti implant, compared with normal cortical bone, upregulation of RUNX2 and SPARC and down regulation of Collagen 1 gene was observed. When the newly formed bone around LAM-Cp-Ti implant was compared with

newly formed bone around Cp-Ti implant, relatively low expression of Collagen 1 gene, relatively high expression of RUNX2 gene was observed. There was no significant difference in the expression of SPARC gene in LAM-Cp-Ti.

Chapter 4 summarizes and concludes the present study. LAM-Cp-Ti implant's long-term biocompatibility and osseointegration property were studied in rabbit femur cortical bone defect. From our study, we conclude that LAM-Cp-Ti promotes osseointegration in long- term.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

There has been an increase in the worldwide use of biomedical devices and implants to evaluate, treat, augment or replace any tissue or organ of a diseased part of the body. In the current scenario where age-related skeletal disorders and bone defects due to trauma are rising day by day, there is an urgent need to develop orthopedic implants which will serve specific conditions. For developing an ideal bone implant, it is important to understand the biological mechanisms of bone, biomaterial chosen for the implant, and the behavior of biomaterial in relation to bone development and healing properties. Creating a strong integration of bone tissue with an implant is a challenging research and new technologies have been used for this purpose to develop biomaterials with better osseointegration property.

Bone fracture is a feature of everyday life due to accident or aging. When a bone is broken the periosteum (outer surface) and endosteum (inner surface touching the marrow) provide bone-forming cells, which endeavor to bridge the fracture. Bone tissue has a remarkable ability to regenerate and heal itself. However, large bone defects and complex fractures present a significant challenge to the medical community. Current

treatments center on orthopedic implants for structural and mechanical support and to substitute long bone defects. The orthopedic implant is a device surgically placed into the body and is designed to restore function by replacing or reinforcing the damaged structure. The main function of orthopedic implants is to provide mechanical and structural support, integrate with the damaged tissue and provide biological cues to promote healing. The implant should be lightweight, mechanically fit and most importantly biocompatible. The determination of biocompatibility of orthopedic implant involves detailed characterization of the material and extensive testing, first at the cell or tissue level and then in in-vivo animal models and ultimately in human clinical trials. The design and use of biocompatibility testing protocols are provided by a variety of professional and regulatory organizations, including American Society for Testing and Materials (ASTM), International Organization for Standardization (ISO), National Institutes of Health (NIH) and Food and Drug Administration (FDA).

Osseointegration is a biochemical phenomenon where rigid fixation of the implant is achieved and is maintained in bone during functional loading. An implant is said to be osseointegrated when there is lack of movement between the bone and implant following healing period under normal conditions. The quality of osseointegration between bone tissue and the implant mostly depend on the characteristics of the material. Materials that are used for biomedical applications are commonly referred to as biomaterials. The characteristic properties that a biomaterial should possess include biocompatibility, bioadhesion, biofunctionality and corrosion resistance. Currently used biomaterials for orthopedic applications include metals (such as 316L stainless steel (316LSS), cobalt chromium (Co–Cr) alloys and titanium and its alloys.), ceramics and polymers.

Titanium is the benchmark material used for orthopedic implants. Selection of titanium is determined by a combination of its most favorable characteristics including better osseointegration property, biocompatibility, bioinert nature, corrosion resistance, mechanical stability and compatibility with magnetic resonance and computed

tomography imaging procedures. Titanium for orthopedic implants has conventionally been used in the commercially pure form. They are available in a range of commercially pure grades depending on the amount of impurity. Commercially pure titanium is fabricated by conventional methods such as casting, powder metallurgy, and machining. Traditional manufacturing techniques typically involve considerable material and energy expenditure and make the fabrication processes tedious and time intensive. In addition, orthopedic implants are required to conform to the complex geometry of human bones and joints which makes their fabrication using conventional methods difficult. Osseointegration of bone implants can be improved by increasing the porosity of implants which can promote bone ingrowth into the metal framework, giving a strong mechanical interlocking between the implant and the bone. Because of these considerations, there is a demand for new fabrication methods, with the aim of obtaining porous titanium framework, with controlled porosity, pore size, and localization.

Titanium processed via advanced powder manufacturing routes, such as additive manufacturing is receiving increased attention for mass-customization in the product line. Laser additive manufacturing is one of the advanced additive manufacturing processes that are capable of fabricating engineering components directly from a solid model. Laser sintering (LS), laser melting (LM), and laser metal deposition (LMD) are presently regarded as the three most versatile laser-based additive manufacturing (AM) processes. Laser-based AM processes generally have complex physical and chemical metallurgical nature, which is material and process dependent. The major advantages include freedom of design, which enables the implant to be designed according to a bone defect and is prepared in less time. Thus, using laser additive manufacturing technique fabrication of functionally designed Titanium implants with graded porosity has a new domain with immense potential finding application in orthopedic implants.

This thesis will take a systematic approach to analyze the effects of modified titanium surfaces on the process of osseointegration, after long-term implantation in a rabbit

model. Improving the understanding of how the process of osseointegration is influenced by alterations in the surface characteristics of titanium implants will hopefully contribute to improvements in implant design, ultimately producing implants which will osseointegrate quickly and efficiently in both routine uses and in more challenging clinical situations. This work was focused on laser additive manufactured titanium implants to determine whether this new technology is as good as or better than conventionally used commercially pure titanium implant, after long-term implantation studies in a rabbit model.

1.2 REVIEW OF LITERATURE

Orthopedic implants are used in patients to replace voids in the fractured bones created during accident or trauma. The aim of current implant research is to design implants that can induce controlled, guided, and rapid healing. In addition to the acceleration of normal wound healing, implants should result in the formation of an interfacial layer and bone matrix with adequate biomechanical properties. In order to achieve this, a better understanding of events at the bone-implant interface and the effects of biomaterials on bone and its cells is needed. Such knowledge is essential for developing strategies to optimally control osseointegration. The natural ability of the human body to adapt with the implant material allows the healing and integration of the inserted implant with the bone. The process that allows this integration is called osseointegration (Osseo-“bone” integration). In this process, the body allows the growth of natural bone into the inserted implant and forms a well-formed structure. This restores the original strength at the implant site. An implant is said to be osseointegrated when there is lack of movement between the bone and implant following healing period under normal conditions. The quality of osseointegration between bone tissue and the implant mostly depend on the characteristics of the material. The concept of osseointegration was first put forward by

Branemark. [Branemark, 1983] Various factors determine the longevity of implant and progress towards osseointegration, including the implant's form, material properties, surface characteristics, mechanical properties, and most importantly its biocompatible nature [LeGeroset al., 1993]. The final goal of osseointegration is to reach an interface matrix, equivalent to the bone in structure, composition and biomechanical properties, to withstand early mechanical loading [Puleo et al., 1999]. Osseointegration was defined as a biomechanical phenomenon in which clinically asymptomatic rigid fixation of the implant has been achieved and maintained in bone during functional loading [Albrektsson & Johansson, 2001]. Biocompatibility is fulfilled when the functionality of the implant is achieved without eliciting a foreign body reaction within the tissue [Niinomi et al., 2008]. Knowledge of ossification mechanisms are important for understanding the biological response to endosseous implants. The bioresponse of an implant can be grouped into:

- Biotolerant: implant is not rejected from the bone tissue but it is surrounded by a fibrous connective tissue (gold, Co- Cr, stainless steel)
- Bioinert: osteogenic cells migrate to the surface of the implant and establish de novo bone formation (Titanium (Ti) &its alloys)
- Bioactive: new bone formation will be formed around the implant resulting in bond formation with bone and implant

1.2.1 BIOMATERIALS

Biomaterials play an indispensable role in medicine— to support, enhance, or replace damaged tissue or a biological function. The modern field of biomaterials combines medicine, biology, physics, and chemistry, and more recent influences from tissue engineering materials science [Nathaniel et al., 2009]. The field has grown significantly in the past decade due to discoveries in tissue engineering, regenerative medicine, and more. Metal has boundless use in the manufacturing of orthopedic implants in a multitude of different forms. Different materials as diverse as ivory, wood, rubber, acrylic, and Bakelite have been used in the manufacture of orthopedic implants throughout history [Chen et al., 2014]. Metals, ceramics, plastic, glass, and even living cells and tissue all

can be used in creating a biomaterial. They can be re-engineered into molded or machined parts, coatings, fibers, films, foams, and fabrics for use in biomedical products and devices including heart valves, hip joint replacements, dental implants, or contact lenses. They often are biodegradable, and some are bio-absorbable, meaning they are eliminated gradually from the body after fulfilling a function. Recently, implants made from iron, cobalt, chromium, titanium, and tantalum are been used due to their mechanical properties and biocompatible nature [Monika et al., 2015].

1.2.2 TITANIUM– MOST PREFERRED BIOMATERIAL FOR ORTHOPEDIC IMPLANTS

Even though many metals have been used as biomaterials, titanium is considered as the benchmark biomaterial due to its excellent mechanical and biological properties Important feature of titanium includes its low electrical conductivity which results in electrochemical oxidation of titanium leading to the formation of a thin passive oxide layer [Quinn et al., 1978]. It has been stated that osseointegration of titanium results due to absence of a negative tissue response [Stanford & Keller, 1991]. The oxide layer formed gives resistance against corrosion. In aqueous environments, titanium and its oxides have a low ion-formation tendency and low reactivity with macromolecules in an aqueous environment. [Tengwall et al., 1992] Various studies done on different implant materials suggested that titanium exhibits a better mechanical and biocompatible nature compared to other conventional materials [Hallab et al., 2003; Eisenbarth et al., 2004] .Thus, the bioinert character of titanium is the reason behind its enhanced bone bonding behavior. Titanium and its alloys exhibit high specific strength [Guo et al 2005] making it an excellent choice for biomedical applications [Sidambe et al., 2012].

Cp-Ti is also referred to as unalloyed titanium and usually contains some trace elements of carbon, oxygen, nitrogen, and iron. These trace elements markedly improve the mechanical properties of pure titanium [MC Cracken 1999] and are found in higher

amounts from Grade I to Grade IV which differs in their oxygen content. Grade IV is having the most (0.4%) and grade I the least (0.18%) oxygen content. Due to its biocompatibility commercially pure titanium is used for various medical applications including surgical tools and orthopedic implants, which can stay in place for up to 20 years. Titanium is non-ferromagnetic; patients with titanium implants can safely be examined with magnetic resonance imaging (Shellock et al., 2011).

The surface of titanium implant plays a pivotal role in determining the biological response of the host bone. The surface of titanium has different characteristics from the bulk form and surface is the only region in contact with the bone. On reaching the surface of the implant the differentiated osteogenic cells start secreting collagen free matrix for mineralization through calcium and phosphate precipitation. It is in this layer initial mineralization occurs and it consists of non-collagenous proteins like osteopontin and bone sialoprotein and proteoglycan [Klinger et al, 1998]. Followed by calcium phosphate precipitation formation, mineralization of collagen fibers takes place and as a result, a non-collagenous tissue is established between the surface of the implant and calcified collagen compartment through contact osteogenesis. The intermediary tissue that is formed is very important for understanding the bonding mechanism between bone and surface. Thus the characteristics of the surface govern the healing mechanisms at the bone-implant interface. A titanium implant material have higher fibrin retention on its surface thus providing osteogenic cells with migration ability to reach the implant surface and starts to produce bone directly on its surface through contact osteogenesis [Davies, 2003]

Even though titanium has superior characteristics compared to other implant materials studies show that osteoconductivity of titanium is lower than calcium phosphate-based bioceramics [Kilpadi et al., 2001]. As a result, various approaches have focused to enhance the bioactivity of titanium to provide higher osteoconductivity by modifying its surface properties In order to improve the biomechanical anchorage of the implant and

for promoting osseointegration at the histological level, the modifications have been done. Modifications such as altering surface topography or coating of titanium with bioactive materials have gained the attention of many scientists, clinicians, and manufacturers throughout the world [Oshida et al., 2007].

1.2.3 SURFACE MODIFICATION OF TITANIUM IMPLANTS

The rough surface topography of implants can finally lead to a better and faster bone response [Buser et al., 2001]. Over the past years, various surface treatments have been studied, which includes sandblasting, grit-blasting, acid-etching, and anodization; deposition of hydroxyapatite, calcium-phosphate crystals etc. to obtain better implant surfaces [Wennerberg et al., 2009]. Several in vitro studies have proved that rough implant surfaces can positively influence bone apposition, compared to smooth surfaces [Novaes, 2010]. Rough surfaces show an increase in molecules adsorption from biological fluids, improving cellular responses, including extracellular matrix deposition, cytoskeletal organization, and tissue maturation. Histological studies from various clinical studies clearly show that rough surfaces can stimulate a faster and effective osseointegration compared to smooth ones [Chiang et al., 2016]

The first initiative to introduce pores in materials was taken by Sosnik. Porous materials used as biomaterials were introduced much later. The earliest works that mention the concept of applying porous metals to osseointegration was done by Weber and White in 1972 [Weber et al., 1972]. Later on, numerous researches on porous materials began in the late 1970s, including porous ceramics [Klawitter, 1974], polymers [Homsy1972, Sauer 1974] and metallic materials [Hirschhorn, 1971] which were demonstrated to be potential candidates for porous implants in animal experimental models. Even though porous ceramics and polymers have been studied as biomaterials, they cannot satisfy requirements under load-bearing conditions [Yoshikawa et al., 2005]. Ceramics portray excellent corrosion resistance, but at the same time, they are fragile due to intrinsic brittleness. Similarly, porous polymeric systems cannot endure mechanical force present

in joint replacement surgery. This impels researchers to focus on porous metals which have the superior mechanical strength and good biocompatibility required for load-bearing applications.

Using these methods, it is not possible to fabricate implants with structure possessing a gradient of porosity perpendicular to the long axis and therefore with a highly porous surface and a highly dense core [Hollander et al., 2006]. Osseointegration of orthopedic implant can be biologically improved by a porous structure with an open interconnected pore system; this system can promote bone ingrowth into the metal framework, giving a strong mechanical interlocking between the fixture and the bone [Traini et al., 2008]. Because of these reasons, there is a demand for new fabrication methods, with the aim of obtaining porous titanium framework, with controlled porosity, pore size, and localization [Ryan et al., 2008]. Therefore, porous titanium and its alloys are emerging as the first choice for biomedical applications. Traditional manufacturing and post-processing methods produce implants with different micro- or nano rough surfaces [Jemat et al., 2015].

Various methods for fabricating porous Ti-based alloys have been studied recently, including investment casting [Yamada et al.,2000], sintering a mixture of titanium powder and space holder method [Wen et al.,2002] sintering loose titanium powder or fiber [Oh et al.,2003], slurry sintering [Li et al.,2005] and laser additive manufacturing. Thus, by increasing the porosity, using any of the above methods, titanium can overcome the mechanical weakness of porous ceramics and polymeric materials. At the same time, they possess interconnected structure to provide space that will allow cells to grow inside biomaterials and body fluid to circulate [Li et al., 2005]. Another frequent problem encountered in the manufacture of orthopedic implants is the challenge of creating the correct shape implants from an appropriate material. In surgery, the patient-specific implant geometries are handcrafted by the surgeon to the best of their ability. However handcrafted implants could have geometric errors that can make them less effective in the

long term. Hence, there is high demand for better manufacturing techniques for developing orthopedic implants.

1.2.4 ADDITIVE MANUFACTURING (AM)

Porous titanium implants have been introduced in orthopedics and dental practice since the end of the 1960s with interesting results [Lueck et al., 1969]. They were generally manufactured using sprays techniques and coating on implant surfaces [Welsh et al.,1971].More recently, different fabrication methods to obtain porous titanium frameworks have been proposed including co-sintering precursor particles, powder plasma spraying over a high-density core titanium fiber, sintering, and solid-state foaming by the expansion of argon-filled pores [Gruner et al.,2001]. Even though many techniques were introduced, none of these methods can manufacture titanium scaffolds allowing complete control over the external shape geometry as well as interconnected pore system [Traini et al., 2008].In the last few decades, 3D printing/additive manufacturing (3DP/AM) technologies have become popular; these allow making physical objects starting from virtual 3D data project, without intermediate production steps, saving time and money [Wang et al., 2016].

1.2.4.1 PRINCIPLE OF ADDITIVE MANUFACTURING

All metal AM technologies are based on the principle of slicing a solid model in multiple layers to create a tool path, uploading these data in the machine, and building the part up, layer by layer, following the sliced model data, using a heat source (laser, electron beam or electric arc) and feedstock (metal powder or wire) [Liou et al., 2007]. Processing of porous Cp-Ti structures is feasible and offers a variety of applications in the medical industry [Lin et al., 2007]. Additive manufacturing of titanium alloys has been well studied because of its numerous applications in the medical industry. Additive manufacturing of titanium offers great potential and finds its best application in producing products that are either highly complex highly customized or where the

quantity needed is small and other production techniques are not cost-effective. The high level of customization available with AM makes this technology well suited for custom-fitting products to individual patients, an important factor in clinical efficacy.

1.2.4.2 LASER ADDITIVE MANUFACTURING (LAM)

Laser additive manufacturing (LAM) process is layer-wise material addition technique which allows manufacturing of complex 3 Dimensional (3D) parts by selective solidification of consecutive layers of powder material on top of each other. The solid structure of the material is achieved by thermal energy supplied by the computer-guided laser beam. It does not require any other post-processing than surface finishing. [Kruth et al., 2010]

The advantages of laser additive manufacturing are geometrical freedom, mass customization, and material flexibility. It can provide consistency over the products entire lifetime. It is also important that processes accuracy and ability to manufacture complex geometrical structures are on a good level [Kruth et al., 2010]

1.2.4.3 PRINCIPLE OF LASER ADDITIVE MANUFACTURING

A thin layer of powder is spread across the build area using a powder-leveling blade in the laser additive manufacturing process. The part building process is performed inside a chamber filled with nitrogen gas in order to minimize degradation and oxidation of the powder material. The powder and the building platform are preheated to minimize the required laser energy and to prevent warping and internal stresses caused by uneven heat distribution during the build [Gebhardt, 2003]

When a thin layer of preheated powder has been formed, a focused laser beam is directed onto the powder bed and moved by using galvanometric mirrors in the scanner so that it melts the material and form a cross-section of the build part. Once one layer is completed, the build platform is lowered by one layer thickness and a new powder layer

is applied by using the recoater blade. After that laser beam scans the next slice of the cross-section. This process is repeated until the whole part is built. Finally, the finished parts are removed from the build platform, loose powder is cleaned off and other finishing operations are performed, if necessary. Finishing operations can include machining, sandblasting, sanding and polishing. [Gibson et al., 2009] The major advantages include freedom of design. The structure can be manipulated to the bone defect. It can be used to fabricate tissue scaffolds that are biocompatible, biodegradable and bioabsorbable.

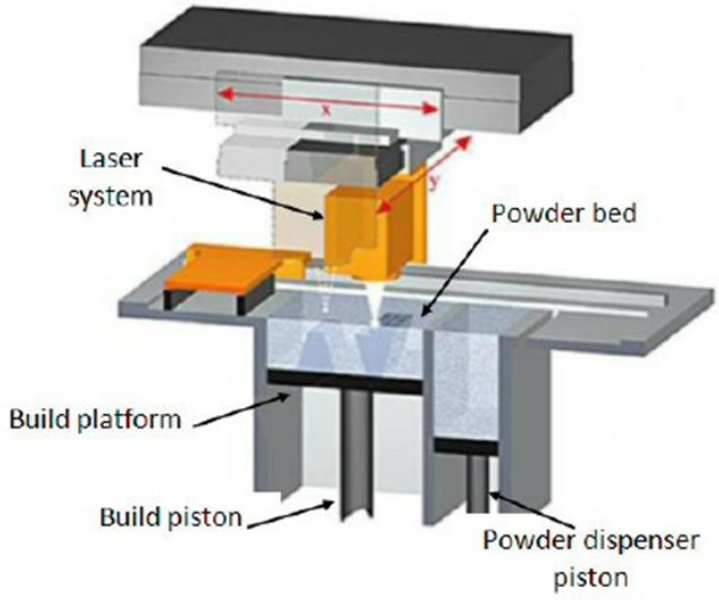


Fig: 1 Basic principle of laser additive manufacturing (LAM) [*Udroiu et al., 2012*]

The physical and chemical properties LAM titanium implants have been extensively studied [Hollander et al., 2006]. In vitro studies conducted by Mangano and coworkers

have investigated the cell response on the surface of LAM implants, examining the formation of a human fibrin clot [Mangano et al., 2009] Witek and coworkers in 2012 have carried out work in laser additive manufactured dental implants in dogs Histologic studies conducted by Shibli and coworkers in LAM dental implants have documented the bone response after the placement of 3DP/AM titanium implants [Shibli et al., 2013] In 2013, Stubinger and coworkers also reported osseointegration in laser manufactured titanium implants in a pilot study conducted in sheep after 8 weeks of implantation. [Stubinger et al., 2013].In the same year, Matena and co-workers performed an in vitro study on laser additive manufactured titanium dental implants [Matena et al., 2013].Last year another study result was published by Naiwen and co-workers in diabetic pig dental implants [Naiwen et al., 2017] However, there are no studies reported on the performance of LAM titanium orthopedic implants after long – term implantation in an invivo model.

1.2.5 BONE

For the proficient cure of abnormalities and injuries of the musculoskeletal system mastery of the complex constantly changing nature of bone is required. Bone is a viable, cellular, highly mineralized and hardest tissue in the body. Bone exists in different shapes which include long bone, like tibia and femur, flat bone, like bones of skull and mandible, and irregular bone, like hip bone. The internal (endosteal) and external (periosteal) surfaces of bone are lined with cellular layers called the endosteum and periosteum, respectively. The interior portion of bone is filled with loose vascular connective tissue, the bone marrow, which contains multipotent stem cells, localized in a defined microenvironment, i.e. niches, [Yin et al., 2006)]. These cells are capable of differentiation along multiple mesenchymal and hematopoietic lineages. The mesenchymal stem cells (MSCs) differentiate into various cell types which include cells from an osteoblastic lineage in addition to chondroblasts, fibroblasts, adipocytes, and myoblasts whereas the hematopoietic stem cells give rise to erythrocytic, leukocytic and

thrombocytic lineages. Osteoclasts, the major bone resorptive cells, are derived from the hematopoietic lineage. Bone is a complex tissue that continually undergoes dynamic biological remodeling, i.e., a coupled process by which osteoclasts resorb mature bone tissue followed by osteoblasts that form new bone and maintain healthy homeostasis of bone [J.D. Currey,2002]. A unique feature of bone is its ability to remodel itself so that it can repair the damage. In chronic cases of bone defects, when the condition is extremely critical, external intervention is needed to supplement the self-healing ability of bone. Important functions of bone include-Providing principal support for the body and attachment sites for organs, taking part in hemopoiesis, act as a reservoir for calcium and other minerals, providing a rigid skeleton for muscle attachment and constitute a system of levers that turn muscle contraction into powerful movements

1.2.5.1 CELLULAR COMPONENTS OF BONE

Cellular components of bone include:

1.2.5.1.1 OSTEOBLASTS: Located along the surface of the bone and are cuboidal in shape. Their function is to form a mineralized organic matrix, facilitate template for bone mineralization. Their morphological characteristics are similar to protein synthesizing cells with the presence of RER (Rough Endoplasmic Reticulum), Golgi apparatus and other secretory vesicles. As osteoblasts are polarized cells, they secrete osteoid towards bone matrix [Damonlis et al 1997]. Osteoblast originates from mesenchymal stem cells. Differentiation of mesenchymal stem cells to osteoprogenitor lineage requires expression of specific genes like Runt-related transcription factor 2 (RUNX2), Dlx5, osterix, Bone Morphogenetic Proteins (BMP). RUNX2 is said to be the master gene of osteoblast differentiation [Fakhry et al 2013]. Combination of high alkaline phosphatase abundance secretion of type 1 collagen and non-collagenous proteins like osteocalcin and bone sialoprotein are its characteristics. Mature osteoblasts can undergo apoptosis and result in osteocytes or bone lining cells. [Parfitt et al, 1990]

1.2.5.1.2 OSTEOCYTE: The osteoblast left behind in newly formed osteoid is osteocyte. Majority of bone cells are osteocytes they are long-lived cells with a life expectancy of up to 25 years [Franz et al, 2006]. Osteocyte shows dendritic morphology and is located within lacunae surrounded by mineralized bone matrix. Osteocytes are also derived from mesenchymal stem cells lineage through osteoblast differentiation. Four recognizable stages that it undergoes include osteoid, osteocyte, pre-osteocyte young osteocyte and mature osteocyte [Franz et al, 2006]. At the completion of bone formation cycle, a subpopulation of osteoblasts become osteocytes and gets incorporated into the bone matrix, during this stage cell undergoes many transformations including decreasing of organelles such as RER, Golgi apparatus. Protein synthesizing capacity of the cell will also gradually get decreased. They function as professional mechanosensory cells of bone and transduce information to the surface of cells of osteoblastic lineage via a network of osteocyte and gap junctions.

1.2.5.1.3 BONE LINING CELLS: They are resting osteoblast. These are flat shaped quiescent cells that cover the surface of bones where neither bone formation nor resorption occurs [Miller et al.1989]. Cells have thin and flat nuclear profile and its cytoplasm extends along the surface of bones and has only a few cytoplasmic organelles such as RER and Golgi [Miller et al.1989]. The physiological status of bone determines the secretory activity of bone. These cells have a role in osteoclast differentiation, producing osteoprotegerin mineral homeostasis, redifferentiation to osteoblast and also have a regulatory role because they participate in bone resorption and bone formation.

1.2.5.1.4 OSTEOCLASTS: Osteoclasts are large multinucleated cells. They originate from the pluripotent stem cells of bone marrow, which generate blood cells. They are related to the monocyte-macrophage lineage and diverge from the monocyte precursors. They are the primary cells involved in bone resorption. Microscopic examination of osteoclasts reveals that resorption is carried out by the specialized surface of the osteoclast. This is a section of the membrane which is in close apposition to the

mineralized surface of the bone. This membrane is highly convoluted and forms a ruffled border. At the edge of the ruffled border, there is a ring of a membrane known as the sealing zone, which adheres tightly to the bone and seals the resorption space. This whole configuration forms a closed space, like a pouch on top of the bone, inside which resorption takes place [Gideon, 1992]. The ruffled border membrane and the transition zone between the ruffled border and the sealing zone allow the secretion of hydrolytic enzymes and the internalization of degraded bone matrix products [Matthew et al., 2014]. For bone remodeling to occur, osteoclast precursors are recruited to the bone surface, where they undergo proliferation followed by differentiation and fusion into mature, multinucleated cells.

1.2.5.1.5 EXTRACELLULAR BONE MATRIX

The bone matrix forms a complex and structured framework that give mechanical support and help in maintaining bone homeostasis. It is composed of an organic matrix and inorganic salts. [Boskey et al.2002]. A major part of the organic matrix includes collagenous proteins, 90% of which includes type 1 collagen. Remaining portion contains noncollagenous proteins including osteocalcin, osteonectin, osteopontin and fibronectin, Bone Morphogenetic Proteins (BMPs), growth factors and bone sialoprotein. It also contains small leucine-rich proteoglycans such as biglycan, lumican [Sornay et al., 2007]. Organic components of bone resist tension, whereas mineral components resist compression. Inorganic material of bone consists predominantly of phosphate and calcium ions apart from these it also contains bicarbonate, sodium, potassium [Downey et al., 2006]. It also releases various chemical mediators that have a role in bone cell activity and bone remodeling [Green et al., 1995]

1.2.5.2 BONE FORMATION

In the early stages of embryonic development, the embryo’s skeleton consists of fibrous membranes and hyaline cartilage. By the sixth or seventh week of embryonic life, the actual process of bone development, ossification (osteogenesis), begins. There are two

osteogenic pathways— intramembranous ossification and endochondral ossification—but in the end, mature bone is the same regardless of the pathway that produces it.

Intramembranous ossification takes place during embryonic development in cranial bones; parts of mandible, maxilla and some of the facial bones and clavicle are formed through intramembranous ossification [Mark et al., 2002]. It is an essential process during natural healing of bone fractures. In intramembranous ossification, a group of mesenchymal cells within the vascularized area of the embryonic connective tissue, and the hematoma of the fracture site proliferates forming mesenchymal condensations which differentiate directly into osteoblasts.

Intracartilaginous (Endochondral) ossification occurs during embryonic development of long bones and postnatal growth of long bones and mandible. It also forms a part of the natural healing process of bone fracture. It begins with the formation of a cartilage-like structure from mesenchymal condensation. Mesenchymal cells instead of differentiating into osteoblasts undergo division and differentiate into chondroblasts which then progressively gets embedded within their matrix, and are then called chondrocytes. Chondrocytes then undergo well-ordered and controlled phases of cell proliferation, maturation and secrete vascular endothelial growth factor and bone morphogenetic proteins that induce the invasion of blood vessels, hematopoietic cells and osteoprogenitor cells which results in the replacement of the cartilaginous matrix by trabecular bone.

1.2.5.3 BONE REMODELING

Bone remodeling is an active and dynamic process which maintains the integrity of skeleton through the balance activity of its cells-the bone forming osteoblast, which produces organic matrix and aids its mineralization [Karsenty et al.,2009], bone degrading osteoclast, which dissolves bone mineral and its extracellular matrix [Teitelbaum et al.,2007], osteocyte which act as a mechanosensor [Bonewald and Johnson, 2008] and bone lining cell which has role in coupling bone resorption to bone

formation [Everts et al.,2002]. The phenomenon of bone remodeling was explained by Frost [Frost, 1990]. The cycle of bone remodeling is carried out by the Basic Multicellular Unit (BMU) comprising a group of osteoclasts and osteoblasts. Bone remodeling is accomplished according to the following phases:

1.2.5.3.1 Activation Phase

Whenever changes happen in the bone such as a micro-fracture or an alteration of mechanical loading, chemical factors, including Insulin Growth Factor-I (IGF-I), Tumor Necrosis Factor-α (TNF-α), Parathyroid Hormone (PTH), Interleukin-6 (IL-6) are released in the bone microenvironment. These are sensed by the osteocytes and they activate the lining cells which are quiescent osteoblasts. As a result, lining cells, increase expression of Receptor Activator of Nuclear κB Ligand (RANKL), which in turn interacts with its receptor Receptor Activator of Nuclear κB (RANK), expressed by pre-osteoclasts. RANKL/ RANK interaction triggers pre-osteoclasts fusion and differentiation toward multinucleated osteoclasts.

1.2.5.3.2 Resorption Phase

Once differentiated, osteoclast polarizes, adheres to the bone surface and starts to dissolve bone. This requires two steps:

- Acidification of the bone matrix to dissolve the inorganic component.
- The release of lysosomal enzymes, such as cathepsins K, MMP9 for the degradation of the organic component of bone.

Once accomplished their function, osteoclasts undergo apoptosis. This is a physiological consequence needed to avoid an excessive bone resorption.

1.2.5.3.3 Reverse Phase

Reversal phase begins when mononuclear cells appear on the surface of the bone. They are macrophage-like cells with a likely function of removal of debris produced during

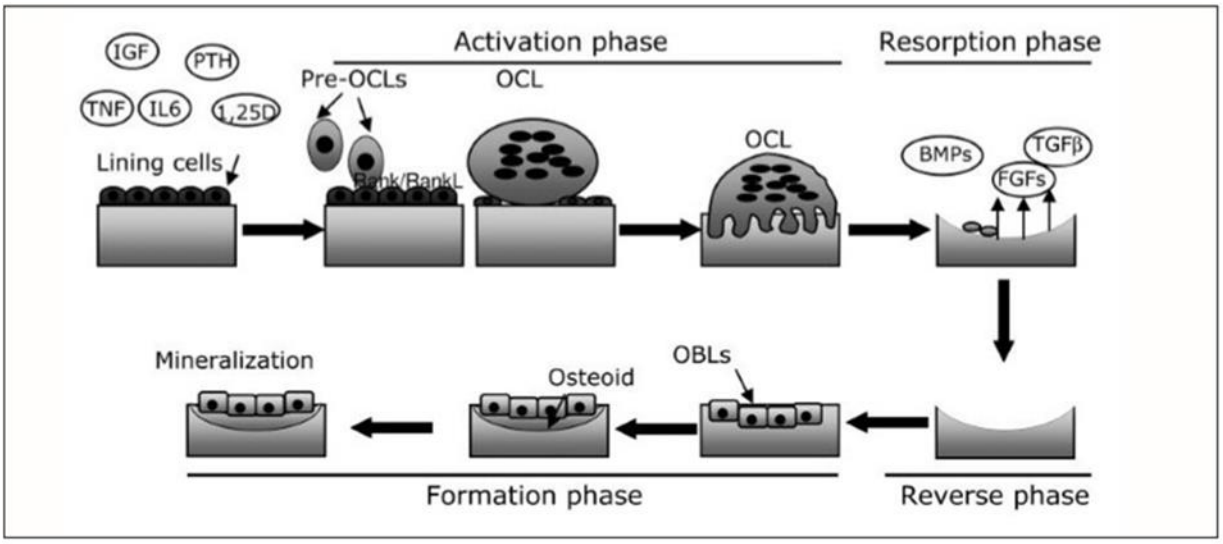
matrix degradation. These cells prepare the surface for new osteoblasts to begin bone formation and provide signals for osteoblast differentiation and migration.

1.2.5.3.4 Formation Phase

Bone matrix resorption leads to the release of several growth factors including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transforming growth factor β(TGF β), which are likely responsible for the recruitment of the osteoblasts in the reabsorbed area. Once recruited, osteoblasts produce the new bone matrix they promote its mineralization, thus completing the bone remodeling process. Unbalance between the resorption and formation phases mirror an incorrect bone remodeling, which in turn affects the bone mass, eventually leading to a pathological condition.

The stages of the remodeling cycle have different durations. Resorption probably continues for about 2 weeks while the reversal phase may last up to 4 or 5 weeks and formation can continue for 4 months until the new bone structural unit is completely created [Matthew et al., 2014].

Bone remodeling helps to repair micro damages in the bone matrix by preventing the accumulation of old bone. It also has a role in maintaining plasma calcium homeostasis. The regulation of bone remodeling is both systemic and local. The major systemic regulators include parathyroid hormone (PTH), calcitriol, and other hormones such as growth hormone, glucocorticoids, thyroid hormones, and sex hormones, growth factors



such as insulin-like growth factors (IGFs), prostaglandins, tumor growth factor-beta (TGF-beta), bone morphogenetic proteins and cytokines. Local regulation of bone remodeling involves a large number of cytokines and growth factors that affect bone cell functions. Furthermore, through the RANK and osteoprotegerin (OPG) system the processes of bone resorption and formation are tightly coupled allowing a wave of bone formation to follow each cycle of bone resorption, thus maintaining skeletal integrity [Rucci N, 2008]

Fig: 2. Schematic representation of the Bone Remodeling Cycle. The remodeling cycle involves five stages: activation; resorption; reversal; formation; (Pre-Osteoclasts –(pre-OCLs); osteoclast (OCL); osteoblast; (OBLs) [*Rucci et al., 2008*]

1.2.5.4 SIGNALING PATHWAYS IN BONE REMODELLING

Cellular damage activates bone remodeling by triggering cellular communication. Remodeling commences with the initiation of osteoclast formation, osteoclast-mediated bone resorption, a reversal period, and then a long period of bone matrix formation mediated by osteoblasts, followed by mineralization of the matrix [Sims et al., 2008]. Cellular signaling provokes osteoclast promoting cells and leads to resorption of bone. Resorption leads to the death of osteoclast cells and initiates the differentiation of osteoblast cells. Finally, osteoblast formation promotes bone formation or osteogenesis. Thus an old bone area regenerates into new bone [Nakahama et al., 2010]. Regulatory factors for osteoblastic phenotype include the essential transcription factors, RUNX2/Cbfa-1 and osterix/SP7 and major signaling pathways, bone morphogenetic protein (BMP), Wnt and notch as well as other growth factor-mediated kinase signaling pathways [Taipaleenmati et al., 2012].RANKL-OPG, Wnt, and BMP pathways have been identified as the classic pathways in the process of bone remodeling [Sun et al., 2016].

RUNX2 plays an essential role in both osteoblast differentiation and expression of osteoblast-specific genes [Komori et al., 1997; Otto et al., 1997]. Further evidence for the

involvement of RUNX2 in osteoblast differentiation came from research conducted by Ducy and colleagues [Ducy and Karsenty, 1995; Ducy et al., 1997]. They investigated the mechanisms of osteoblast-specific gene expression by analyzing the osteocalcin gene, the most osteoblast-specific gene [Ducy and Karsenty, 1995]. The key role of RUNX2 in osteoblast differentiation has been substantiated by the findings that RUNX2 regulates the expression of several osteoblast marker genes in osteoblasts and also induces expression of osteoblast marker genes osteocalcin, collagen type I alpha 1 (Col1α1), bone sialoprotein, and osteopontin in non-osteoblastic RUNX2 is the transcription factor that induces the commitment of mesenchymal stem cells to osteogenic lineage and acts upstream from the other osteoblast-specific transcription factor OSTERIX and other specific osteoblastic genes such as Secreted Protein Acidic and Rich in Cysteine (SPARC) (Osteonectin), Osteopontin, and Type I Collagen [Valenti et al.,2016]. This prominent pathway is regulated by Wnt signaling. Osteonectin is also known as SPARC is produced by osteoblast cells during osteogenesis. It plays a major role in bone development and mineralization [Humphrey et al., 2013]. It binds to Collagen and promotes mineralization [Termine et al., 1981]. Collagen is the main protein component of bone matrix. The reduced level of these proteins may lead to bone loss. So they are required for maintaining the structural integrity of bone. Understanding structural-functional and molecular biology of bone has a crucial role for the better comprehension of bone tissue as a multicellular unit and a dynamic structure [Rinaldo et al, 2015]

1.2.5.5 ANIMAL MODEL FOR ORTHOPEDIC IMPLANTS

Animal models may closely represent the mechanical and physiological human clinical situation. Animal models help in the evaluation of materials for long time durations and in different tissue qualities (e.g. normal healthy or osteoporotic bone) and ages. With the help of animal models, tissues in the near vicinity of the implant and in far locations can be studied, which is relevant for the study of toxic effects of implants. In the case of human patients, wear debris from implants has been reported to travel into distant organs

such as liver and spleen [Urban et al., 2000]. But it should be kept in mind that animal model study is only an approximation, with each animal model having unique advantages and disadvantages. At present, there are numerous animal models available for the testing implant. Animal fracture models allow researchers to understand clearly the physiology of bone-healing and to improve the rate, speed, and quality of fracture-healing [Padhraig et al., 2008].

The laboratory rabbits (Oryctolagus cuniculus) belong to the family Leporidae of the order Lagomorpha [Okerman, 1989]. The common strain used is the New Zealand White (5–6 kg). Rabbits are taken as the first-hand choice for implant studies because of their easy handling, size, short lifespan, and economic aspects of purchasing and maintaining them. At around 6 months of age, rabbit reaches their skeletal maturity [Stefan et al., 2013]

1.2.5.6 TISSUE RESPONSE TO IMPLANTATION

Whenever a foreign body such as an implant is inserted into the body, there results in immune reaction against the foreign body and it also results in injury to the surrounding host tissues. As soon as the implant is placed, the implant surface first gets in contact with the blood that is arising from the damaged blood vessels near the implant cavity. After some time surface of implant gets completely covered with thin layer of serum proteins. This is same for all implant materials, but the surface characteristics of implant materials have a major impact on the structure and conformation of protein layer [Dee et al., 2002]. Soon after protein adsorption, the surface of implant become associated with thrombocytes following these coagulation mechanisms takes place. As a result of thrombocyte aggregation and degranulation on the implant surface cytokines, vasoactive factors (serotonin and histamine) gets released from cytoplasmic granules of thrombocytes. These chemical mediators trigger proliferation and migration of various cells thereby directing the implant healing mechanisms [Dereka et al., 2006]. Polymorphonuclear neutrophils (PMNs) are the first group of cells that take part in

inflammatory response. PMNs dominate the bone-implant interface for the first two days. When bacteria and endotoxins are not present at the interface of the implant, number of PMNs tends to decrease During the second day of healing, migration of monocyte and macrophage accumulation starts to occur [Davies, 2003] PMNs and macrophages help in removing dead cells, extracellular matrix residues and bacteria. Other than inflammatory function, macrophage has a role in the expression of cytokines which help in providing signals to stimulate the recruitment of osteogenic and endothelial progenitor cells for next proliferative phase. Important events that occur at the inflammatory phase include macrophage activity, the release of vasoactive amines, and infiltration of thrombocyte and leukocytes, coagulum and fibrin network establishment. The first phase of inflammation can sometimes extend up to five days, coagulum is removed by PMNs followed by monocytes angiogenesis also takes place [Stanford et al., 1991]

As a result to the response to hypoxia and acidic nature of the bone-implant interface, growth factors expressed by macrophages endothelial cells stimulate the growth of new capillaries into the fibrin network [Schliephake et al., 2002].In this way, proliferation maturation and organization of endothelial cells to new capillary tubes takes place by providing oxygen and nutrients to the newly formed bone tissue at the bone-implant interface. The response of blood cells inside the fibrin-based structural matrix has a prime impact on the healing mechanisms at the bone-implant interface. The capacity of osteogenic cells to proliferate and migrate around the implant has also a role in the quality of bone healing. Studies conducted by Meyer et al, 2004, have indicated that osteoprogenitor cells get attached to the implant surface after the first day of implantation.

1.2.5.7 BONE-IMPLANT INTERFACE EVENTS

At the first day of implant placement, layer of water molecules form around implant which facilitates protein and other molecule adsorptions on the implant surface [Shard et al.,2006] Within thirty seconds to several hours after the implant placement, the surface

will be coated with a layer of intercellular matrix proteins [Thevenot et al.,2008] These proteins initially come from blood and interstitial fluid in wound location and then derive from cell activity in the area around the implant cells interact with the implant surface occurs via a protein layer, which is initiated by cell adhesion, migration, and differentiation that lasts for several hours or days [Wilson et al.,2005] This phase is finely adjusted with extracellular matrix (ECM) proteins, cell surface binding and cytoskeleton proteins, chemical characteristics, binding topography, and chemical ion release [Ratner et al.,2004] During the third day, osteoblast transcription factors of RUNX2 and Osteopontin are activated by the cells around the implant. By the 4th day, the created necrotic bone within the surgery is reabsorbed and a certain interface zone is formed. In the 5th day new bone formation and the presence of alkaline phosphatase activity are seen which indicates the beginning of mineralization and matrix remodeling [Colnot et al.,2007] By the end of first week, the cohesion of bone matrix on the surface of implant could be recognized easily, ECM becomes engaged in the surface. Up to the 16th day, the implant surface becomes fully and abundantly coated with a mixture of mineralized tissues, osteoid, and dense matrix On the 28th day, which is the end of 4th week, the main bone establishes a complete binding along the implant surface and also in the neck, collagen fibers, and osteoblasts create a volume of tissue layer adjacent to the implant. The initial bones are formed woven, which have osteoid in their matrix. At the end of 12th week, the new bone that is formed at the implant surface will be uniformed with a body connection of mature lamellar bone with titanium surface [Depprich et al., 2008]

Surface properties of implant determine the cell-surface interactions. It enhances the process of osseointegration and acts as a good candidate for bone remodeling after damage. Porosity and pore size plays a critical role in osseointegration [Karageorgion et al., 2005]. Porous implants improve anchorage of bone cells by creating a mechanical interlock by favoring bone growth into porous implant structure [Ryan et al., 2006]. Thus

by controlling porosity, osseointegration property of material can be modulated. Laser additive manufacturing technology is a technique in which there is complete freedom in designing implant. It is done through the computer-aided model. This technique enables to design implant according to bone defects and can also have control of porosity. However, only a few clinical studies have investigated the long-term performance of LAM titanium implants. In the present study laser additive manufactured commercially pure titanium (LAM-Cp-Ti) obtained from Raja Ramanna Centre for Advanced Technology (RRCAT) has been evaluated for its biocompatibility and osseointegration capacity after long- term implantation when compared to that of the conventionally manufactured commercially pure titanium rods in relation to its porous structure and topography.

1.3 HYPOTHESIS

The hypothesis of this research work is that Laser Addictive Manufactured Commercially Pure Titanium (LAM-Cp-Ti) implant promotes osseointegration when implanted in femur cortical bone defects in a rabbit model.

1.3.1 AIM

To evaluate the long-term osseointegration and biocompatibility of LAM-Cp-Ti implanted in femur cortical bone defects in a rabbit model.

1.4 OBJECTIVE

- Histopathological evaluation of biocompatibility and osseointegration of LAM-Cp-Ti after six months of implantation
- Gene expression studies of osteogenic genes around implant interface after six months of implantation.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIAL

The implants used for the study were small rods (2mm diameter x 6mm length). Laser additive manufactured commercially pure titanium (LAM-Cp-Ti) implants obtained from Raja Ramanna Centre for Advanced Technology (RRCAT), Indore, Madhya Pradesh served as test and commercially pure titanium (Cp-Ti) (medical grade 4) implants served as control. 10% Neutral Buffer Formaldehyde, RNAlater^{TM} (Thermo Fischer Scientific, USA).

2.2 INVIVO EVALUATION-LONG TERM

New Zealand White rabbit was the animal model used for the study. Five adult New Zealand white rabbits, aged ten months, weighing more than 2.5 Kg, of both sexes, with normal limbs were used for the study. The study protocol was approved by the Institutional Animal Ethics Committee (IEC) (SCT/IAEC-193/Nov/2016/90 dated 29.12.2016) and following Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, conducted the experiments in animals. Animal implantation studies were conducted as per ISO 10993-6 standard. Rabbit femur bones were implanted with titanium pins. In right femur bone Cp-Ti (control) pins were implanted and in left femur, LAM-Cp-Ti (test) pins were implanted.

2.3 EXPLANTATION

Post six months of implantation, animals were euthanized humanely in the carbon dioxide (CO_2) chamber. Femur bones with implant were collected and the implant sites

were identified. For molecular studies, implant and surrounding bone tissue were removed and stored in RNAlater^{TM} .Along with implanted bone; normal bone was taken and kept as a control. For histological analysis, femur bones with implant were collected and stored in 10% neutral buffer formalin.

2.4 HISTOLOGICAL ANALYSIS

2.4.1 MATERIALS

2.4.1.1 REAGENTS: 10% Neutral Buffer Formaldehyde, Alcohol (70%, 80%, 96% and 100%), Acetone (Thermo Fischer, Mumbai), Methyl Methacrylate (MMA) (Merck, Mumbai) Benzoyl peroxide (Merck, Mumbai), 5% Sodium Hydroxide, Potassium Permanganate (Merck, Mumbai) Methylene Blue (Qualigen, Mumbai) Distilled water, Acid Fuschin (Central Drug House, Bombay) Saturated Picric Acid (Nice Chemicals, Cochin), Cyanoacrylate glue (Alteco chemicals, Japan), Stevenel's blue and Van Gieson's Picrofuchsin stain.

2.4.1.2 EQUIPMENT: Vacuum pump (LabConco, USA), Precision saw (Accutom 100, Struers, Denmark), Grinder polisher (Ecomet 3, Buhler, USA), Waterbath (H11210, Leica, USA) Light Microscope (Nikon E 600, Japan).

2.4.2 PROCESSING AND EMBEDDING OF HARD TISSUE

2.4.2.1 HARD TISSUE PROCESSING

Processing was done for hard tissues with an implant for final impregnation with a solid medium to facilitate cutting of sections for microscopic observation. Harvested bone tissue was cleaned and fixed in 10% neutral buffered formalin for 10 days. Bone tissues

were then subjected to dehydration through ascending grades of alcohol at room temperature.

- 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) mixture for 1 day
- 100% alcohol for 1 day

After the tissues were subjected to dehydration they were infiltrated with the washed monomer (MMA). Bone tissues were transferred into two changes of washed monomer and kept in the refrigerator for 4 days.

2.4.2.2 EMBEDDING OF HARD TISSUES IN MONOMER

Embedding solution must be made fresh. Separate embedding bottles were used for each tissue. Each bottle was labeled with an identification number. One gram of recrystallized Benzoyl peroxide was made up to 100 ml using washed monomer. This forms the embedding solution. Embedding solution was poured into an airtight bottle. Bone tissue was removed from the processing bottle and was oriented with cutting surface facing down. The airtight bottle was loosened and placed in the vacuum desiccator for 30

minutes to one hour for removing any trapped air bubbles. The vacuum was slowly removed and the cap was tightened without displacing the position of tissue. The airtight bottle was kept in vacuum for three days to ensure complete evacuation of oxygen in the chamber. Each bottle was then checked for polymerization with a needle. Polymerization gets completed when resin becomes hard. Bottles in which polymerization occurred was broken and labeled with an identification number.

2.4.3 SECTION CUTTING OF RESIN EMBEDDED TISSUES

For getting thin sections (100 µm) of resin blocks, precision saw microtome (Accutom 100, Denmark) was used. Blade thickness, section thickness, blade speed, feed rate, cutting length and the number of sections required was entered before the equipment was operated. Complete sectioning was done till the implant site is finished. Each section contains an implant with surrounding bone tissue. Each section was then fixed on a clean glass slide using cyanoacrylate glue.

2.4.4 GRINDING AND POLISHING

Cut sections were stuck to a glass slide and held using slide holder, the sections were ground to reduce the thickness using different grades of sandpaper in a grinder cum polisher machine (Ecomet 3, USA). This facilitates the sections to be viewed under a microscope. (Nikon E 600, Japan).

2.4.5 STAINING OF RESIN SECTIONS

2.4.5.1 PREPARATION OF STEVENEL'S BLUE STAIN

1.5 g of Potassium permanganate was dissolved in 75 ml distilled water.1 g of Methylene Blue was dissolved in distilled water and poured into potassium permanganate solution. The resulting mixture was then kept in a boiling water bath until the precipitate has dissolved. The solution was allowed to reach room temperature and then filtered. The filtrate is then labeled and kept in a reagent bottle.

2.4.5.2 PREPARATION OF VAN GIESON PICROFUCHSIN STAIN

0.1 g of acid Fuschin was dissolved in 10 ml of distilled water; 100 ml of saturated picric acid was added to 1% acid Fuschin solution and mixed well. The reagent is labeled and stored in a reagent bottle.

Resin sections were stained using Stevenel's blue stain for 5 minutes and washed with hot distilled water to remove excess stain. Van Gieson Picrofuchsin stain was then added to resin sections and kept for 5 minutes at room temperature. Excess stain was removed and sections were observed under a microscope (Nikon E 600, Japan). Microphotographs were captured using the camera attached to the microscope (DS-Ri1 Nikon, Japan).

2.5 MOLECULAR STUDIES

2.5.1 RNA ISOLATION FROM FEMUR BONE

2.5.1.1 MATERIALS

Bone tissue surrounding the implant from test and control samples, normal cortical bone without implant, RNAlater^{TM} (Thermo Fischer, USA), 70% Ethanol (Jebsen & Jessen, Germany), Chloroform (Ranbaxy, Delhi), Isopropanol (Merck, Mumbai), Ultra Pure^{TM} Distilled water (Life Technologies, USA) , mortar and pestle, Liquid Nitrogen (Meera Traders, Thiruvananthapuram) TRIzol^{R} (Life Technologies, USA)

2.5.1.2 EQUIPMENT

Centrifuge (Eppendorf, USA), homogenizor (polytron), Nanodrop Spectrophotometer (Nano Drop Inc. Wilmington, USA)

2.5.1.3 METHOD

The total RNA was extracted by using TRIzol method [Chomczynski and Sacchi, 1987]. Bone tissue surrounding the implant and normal bone tissue without implant was taken out from RNA later and washed in ultra-pure distilled water. Liquid nitrogen was added to sample and was crushed using mortar and pestle. Mortar and pestle were kept cold on dry ice. Fine powder bone was obtained which was transferred to a 50 ml falcon tube.

***Homogenization*:** 1 ml of TRIzol was added to the bone powder and was homogenized using a homogenizer. Homogenized sample was then incubated for 5 minutes at room temperature and centrifugation was done to remove cell debris (1500 rpm), the supernatant was transferred to a new tube

***Phase separation*:** 200 µl of chloroform was then added to 1 ml of sample and are then covered tightly and mixed well by gently inverting for 10 to 15 minutes. After mixing, the sample was allowed to stand for 5 minutes at room temperature. Centrifugation was done at 12000 rpm for 15 minutes at 4^0C . Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colourless upper phase. RNA remains exclusively in the aqueous phase. Colourless upper aqueous layer containing RNA was removed and transferred to a fresh tube.

RNA precipitation: Aqueous phase was separated and 500 µl of isopropanol was added and gently mixed by inverting and was allowed to stand at room temperature for 10 minutes. Centrifugation was repeated at 12000 rpm for 10 minutes and the supernatant was discarded, which leaves the RNA pellet at the bottom.

***RNA wash*:** Tubes were then gently tapped to remove excess isopropanol.1 ml of 70% alcohol was added and was centrifuged at 7500 rpm for 5 minutes at 4^0C.The supernatant was removed and the pellet was dried in orbital shaker incubator for 10 minutes.

***Redissolving RNA*:** The RNA pellet was then resuspended in 25 µl of sterile water.

***RNA quantification*:** The quantity OD 260 nm and purity of total RNA OD260/OD280 nm was measured using 1 µl of total RNA on a Nanodrop.

2.5.2 cDNA SYNTHESIS

The total RNA was immediately subjected to cDNA synthesis using reverse transcription according to manufacturer's protocol. (TaKaRa Cat. #RR037A).Prior to cDNA synthesis, the concentration of RNA in each sample was calculated and 1 µg of RNA from each sample was taken for cDNA synthesis according to the kit protocol

2.5.2.1 MATERIALS REQUIRED

PrimeScript™ RT reagent Kit (TaKaRa Cat. #RR037A), 5X prime script buffer, Prime script RT enzyme mix I, Oligo dT primer, Random 6mers, Ultra Pure™ Distilled water(Life Technologies, USA) Thermal Cycler (Eppendorf, USA)

2.5.2.2 PROTOCOL

Preparation of master mix for cDNA synthesis (10 µl)

REAGENTS	VOLUME
5X prime script buffer	2 µl
Prime script RT enzyme mix I	0.5 µl

Oligo dT primer	0.5 µl
Random 6 mers	0.5 µl
Total RNA	1 µg
RNase free water	As required
Total volume	10 µl

Table 1. Master mix for cDNA synthesis

Thermal cycling conditions for cDNA synthesis

	STEP 1	**STEP 2**	**STEP 3**
TEMPERATURE	37^0C	85^0 C	4^0C
TIME	15 min	5 sec	∞

Table 2. Reaction conditions for cDNA synthesis

The cDNA samples were stored at -20^0C until further real-time PCR analysis.

2.5.3 REAL TIME PCR AMPLIFICATION FOR EXPRESSION OF RUNX2, COLLAGEN 1, AND SPARC

2.5.3.1 MATERIALS REQUIRED: KAPA SYBR® FAST qPCR Master Mix (2X) Kit (catalog: KR0389) PCR grade water, KAPA SYBR FAST qPCR master mix, 10 µm forward primer, 10 µm reverse primer, Template DNA, thermal cycler (QTower 3, Analytik Jena, Germany)

2.5.3.2 LIST OF PRIMERS FOR EXPRESSION STUDIES OF RUNX2, COLLAGEN I AND SPARC

SL.NO	OLIGO NAME	5'----------3' SEQUENCE	BASE PAIRS
1	Collagen I forward	GCAAGAACGGAGATGACGGA	20
	Collagen I reverse	TTGGCACCATCCAAACCACT	20
2	RUNX2 forward	ACCAGTCTTACCCCTCTTACCT	22
	RUNX2 reverse	AGGTGCTGGGCTCTGAATCTG	21
3	SPARC forward	GAAGTAGTGGCCGAAAACCC	20

	SPARC reverse	TGGGGGTGTTGTTCTCATCC	20
4	GAPDH forward	CAACGAATTTGGCTACAGCA	20
	GAPDH reverse	AAACTGTGAAGAGGGGCAGA	20

Table 2. List of list of primers for expression studies of RUNX2, COLLAGEN I and SPARC

2.5.3.3 PROTOCOL

Following was the master mix used for Real Time PCR analysis.

REAGENTS	**VOLUME (µl)**
PCR grade water	8.4
KAPA SYBR FAST qPCR master mx	10
10 µm forward primer	0.4
10 µm reverse primer	0.4
Template DNA	0.8

Total volume	20

Table 4. Master mix for Real Time PCR analysis

The reaction mixture was incubated under the following condition

	STEP1	STEP2	STEP3
TEMPERATURE	95^0C	95^0C	60^0C
TIME	3 min	3 sec	20 sec

Table 5 .Reaction conditions for Real Time PCR analysis

The cDNA copy number of the target gene was normalized to the reference gene, GAPDH, which is a housekeeping gene (gene constitutively expressed in all cells and is independent of treatment protocol). Real Time PCR gene expression measurements were converted to mean fold changes.Relative gene expression levels were calculated using delta-delta Ct-method [Livak et al., 2001]

2.6 STATISTICAL ANALYSIS

Data were analyzed using calculation software [Microsoft Excel, Microsoft Corp., Redmond, WA, USA]. The stastical analysis was carried out for all quantitative data. Student's t-test was used to determine P value so that presence of significant difference

can be found out. Significance is considered at P = 0.05 All the values given in tables are represented by Mean ±SD.

CHAPTER 3

RESULTS AND DISCUSSION

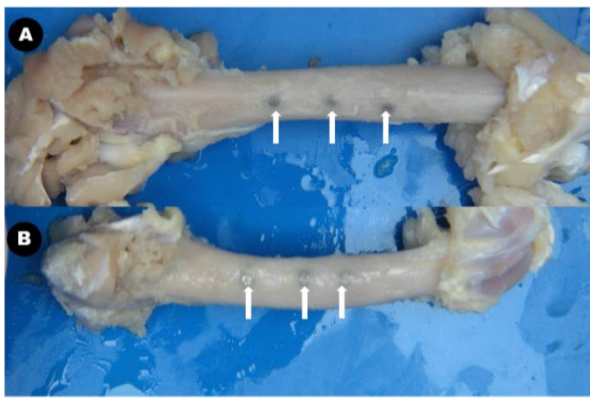
3.1 RESULTS

3.1.1 ANIMAL STUDIES

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. No adverse reaction was observed during this procedure.

3.1.2 GROSS PATHOLOGY OBSERVATIONS

All implants were found at the implant site. No gross abnormalities were detected at the implant site. The titanium pins were intact at the implant site and covered with a thin



layer of new bone on the periosteal surface.

Fig: 3 Explanted- femur bone with LAM-Cp-Ti (A).and Cp-Ti (B) implants (arrow)

3.1.3 HISTOLOGY ANALYSIS

On microscopic evaluation, there was the absence of necrosis and degeneration at implant-bone interface. There was no intervening soft tissue between the implant and bone. New bone formation was observed at the implant interface. Actively secreting osteoblasts were observed in the marrow spaces in multiple focal areas at the interface. New woven bone arising from periosteum and endosteum was observed which is found filling the interface. Bone tissue was found anchoring the pins all around the implant region. Inflammatory cells were absent. Bone formation and remodeling in the Haversian canals were still occurring 6 months after implantation. Histologically, no qualitative difference in bone formation could be seen between test (LAM-Cp- Ti) and control (Cp-Ti) implants.

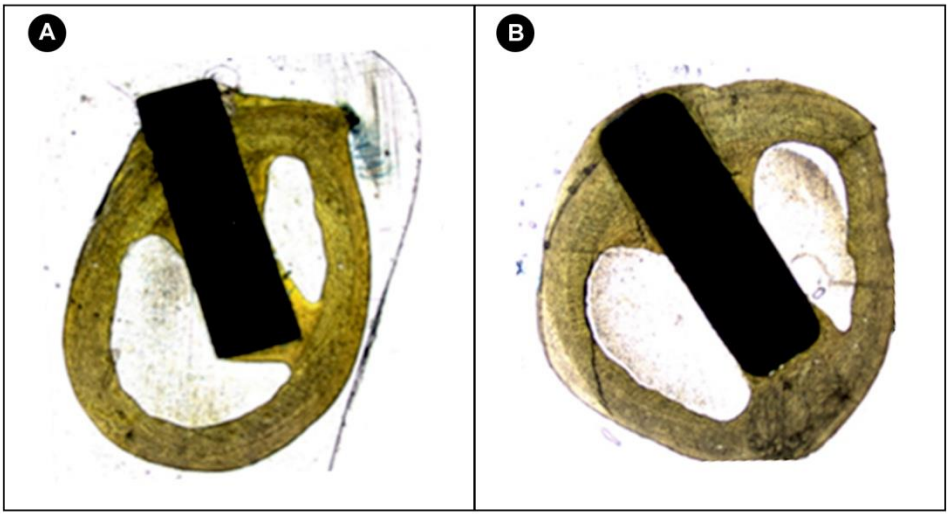
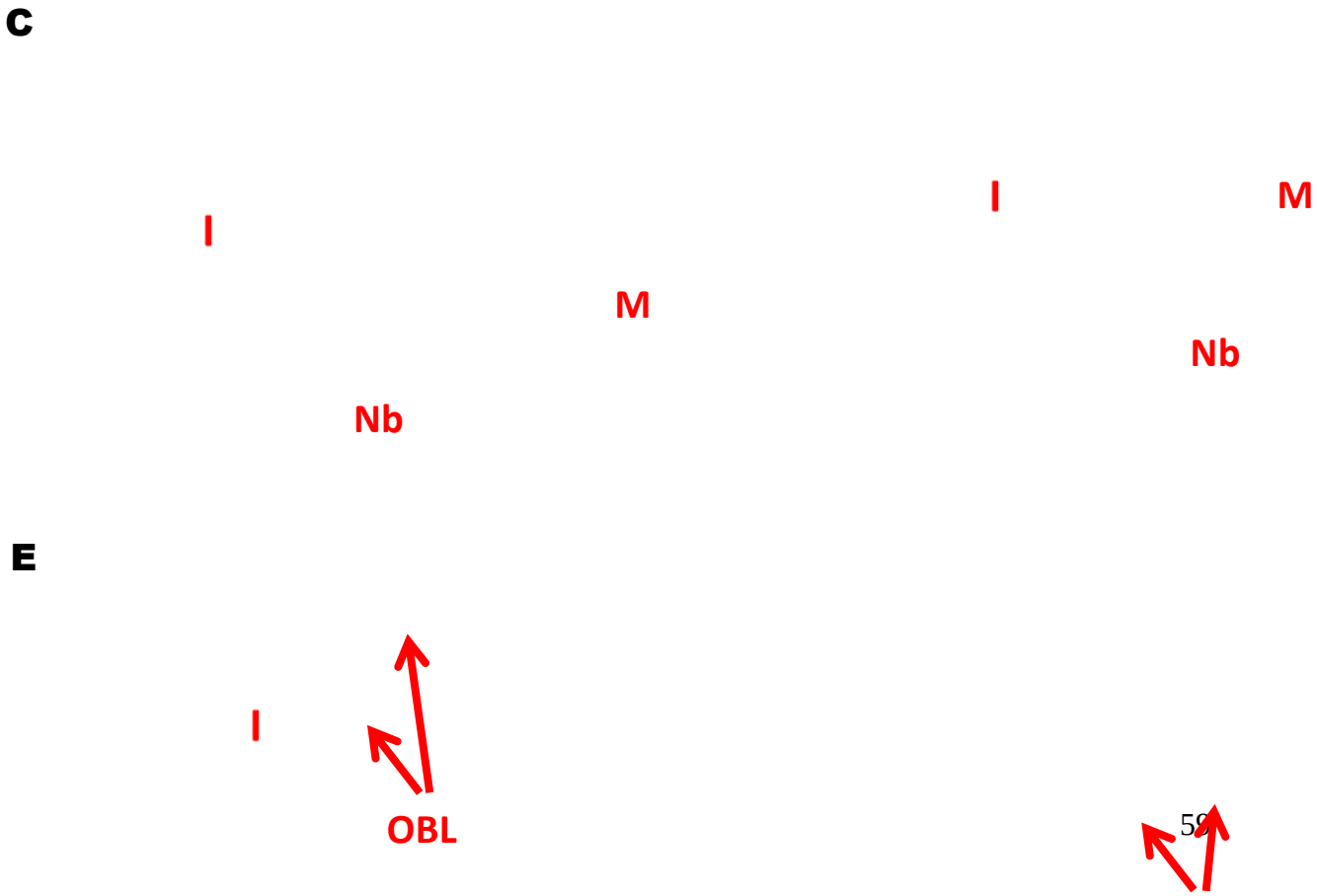


Fig: 4 Femur cross section of LAM-Cp-Ti (A) and Cp-Ti (B) implants (low magnification)



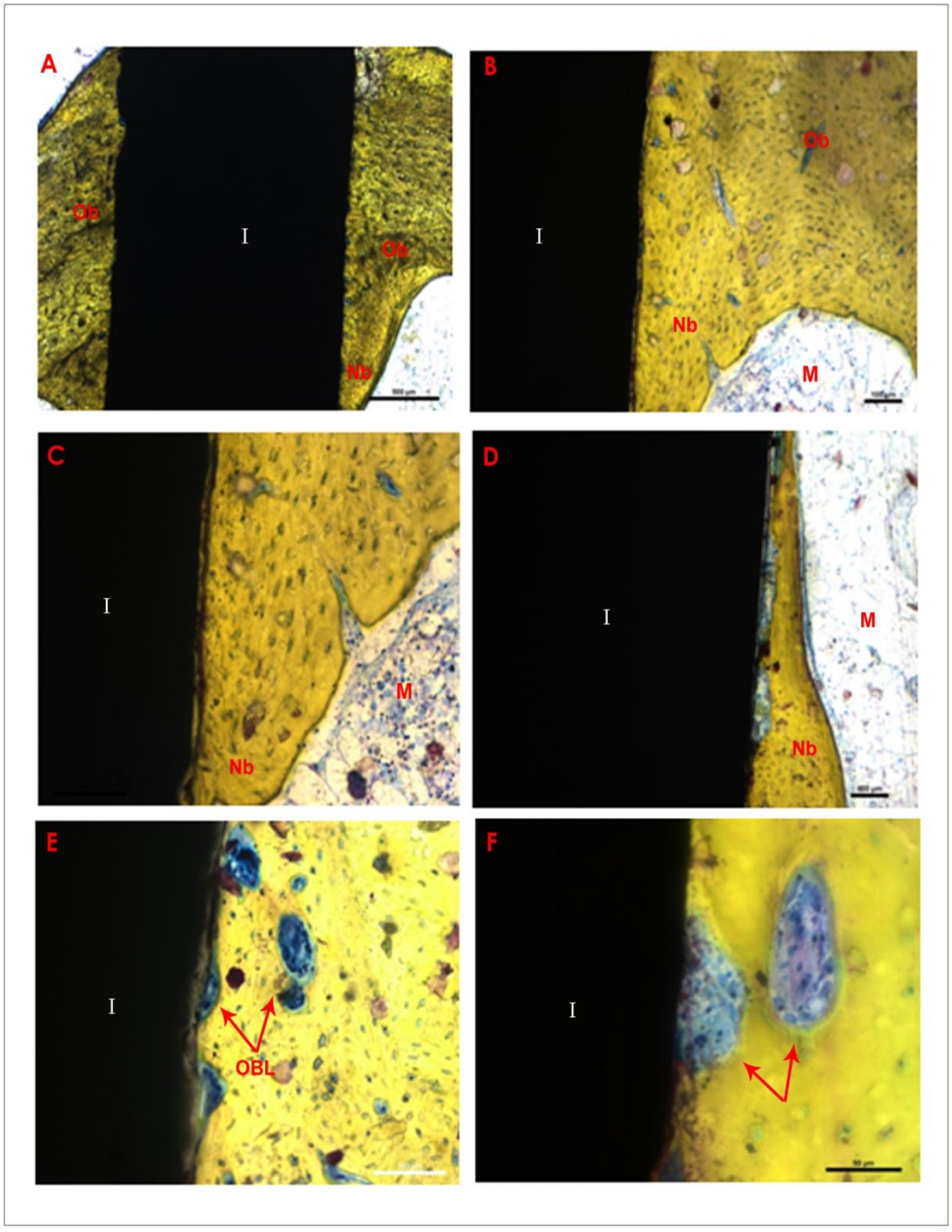


Fig: 5 Histology images of LAM-Cp- Ti implant.

cortical bone (Cb) ,implant (I) marrow (M) ,new bone (Nb) old bone (Ob)osteoblast (OBL) .Interface new bone region with rosette of osteoblast cells activity (arrow).Scale bar:(A,C,E 500µm; B,D 100µm; F 50µm)

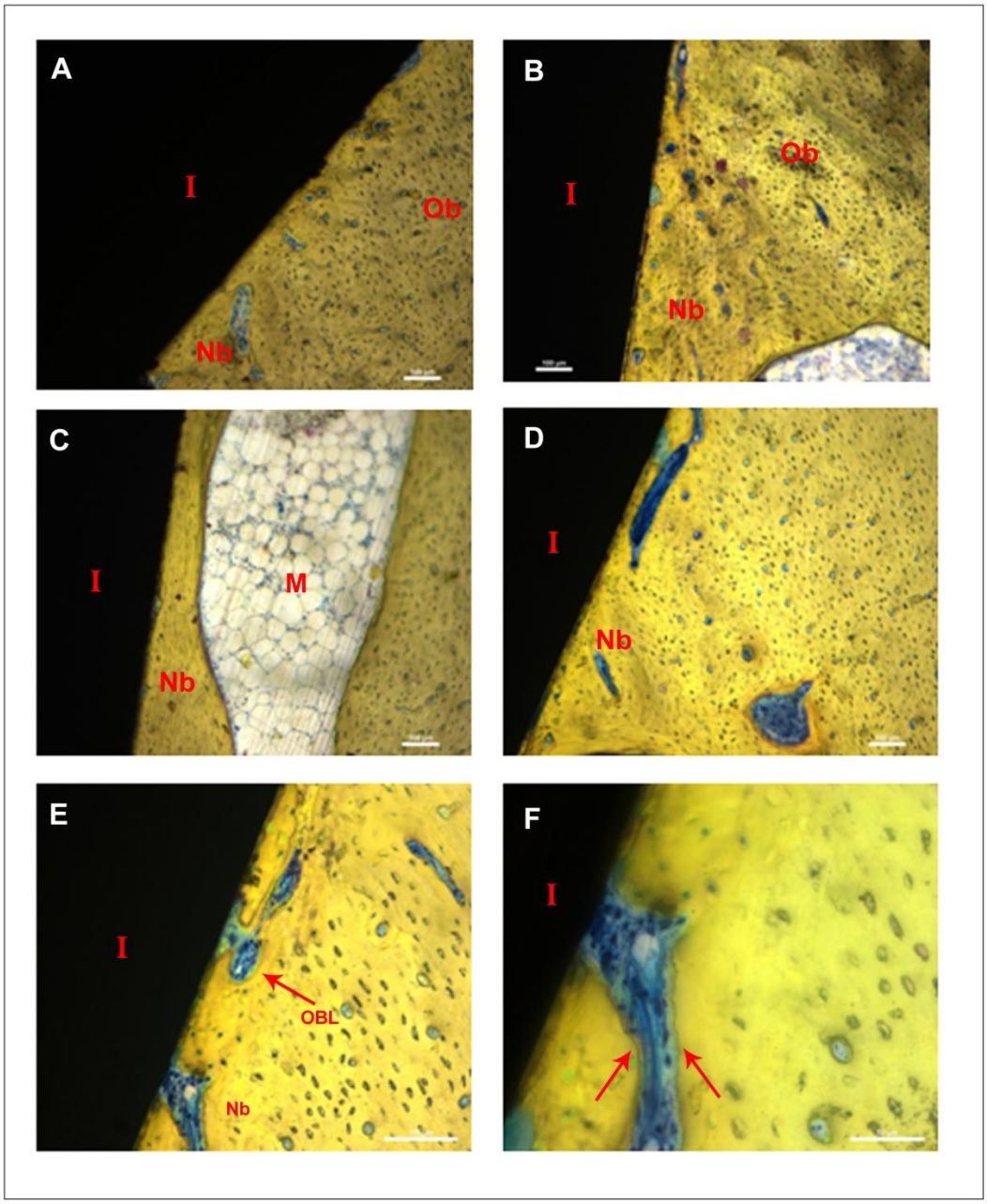


Fig: 6 Histology images of Cp- Ti implant

cortical bone (Cb) ,implant (I) ,marrow (M) ,new bone (Nb) ,old bone (Ob). Osteoblast(OBL)Interface new bone region with rosette of osteoblast cells activity (arrow).Scale bar: A B C 100µm; D,E 500µm; F 50µm.

3.1.4 MOLECULAR STUDIES

3.1.4.1 GENE EXPRESSION PATTERN OF RUNX2, COLLAGEN-I, AND SPARC IN BONE HEALING

The expression of RUNX2, Collagen 1 and SPARC was determined using qRT PCR in terms of fold change. The study was carried out in 5 rabbit samples.

3.1.4.1.1 Laser Additive Manufactured Titanium (LAM-Cp-Ti) vs. normal Bone

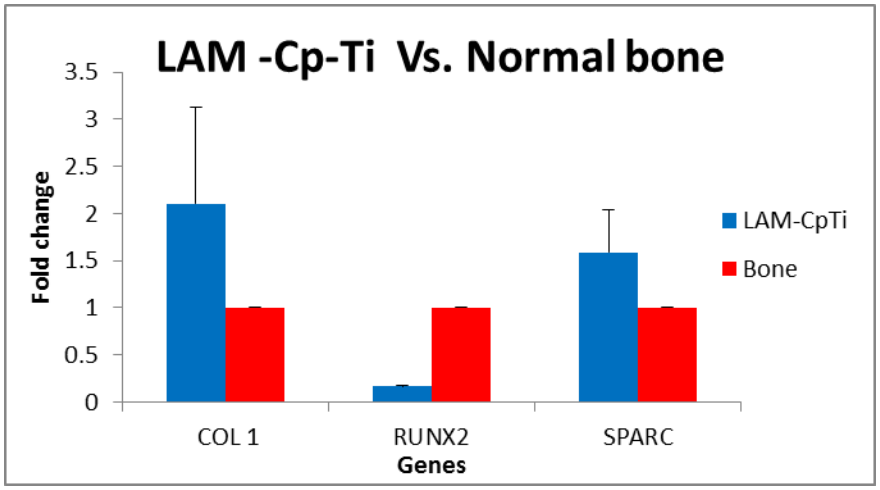


Fig: 7 Graphical representation of RT PCR data depicting the fold change in expression of bone-specific genes (values are represented in mean ±SD (n=5).P value >0.05 Abbreviations: Collagen 1 (COL 1); Runt-related transcription factor 2 (RUNX2) Secreted protein acidic and cysteine-rich (SPARC)

The expression of Collagen 1 and SPARC was found to be up-regulated in bone implanted with LAM-Cp-Ti compared to normal bone. However, the expression of RUNX2 was found to be down-regulated in bone implanted with LAM-Cp-Ti compared to normal bone. The P value was found to be greater than 0.05 showing that there is no significant difference in terms of Collagen 1 and SPARC between LAM-Cp-Ti and normal bone, thus the higher expression of Collagen 1 and SPARC in LAM-Cp-Ti indicates that mineralization phase is continuing which indicates osseointegration.

3.1.4.1.2 Commercially pure Titanium (Cp-Ti) vs. Normal Bone

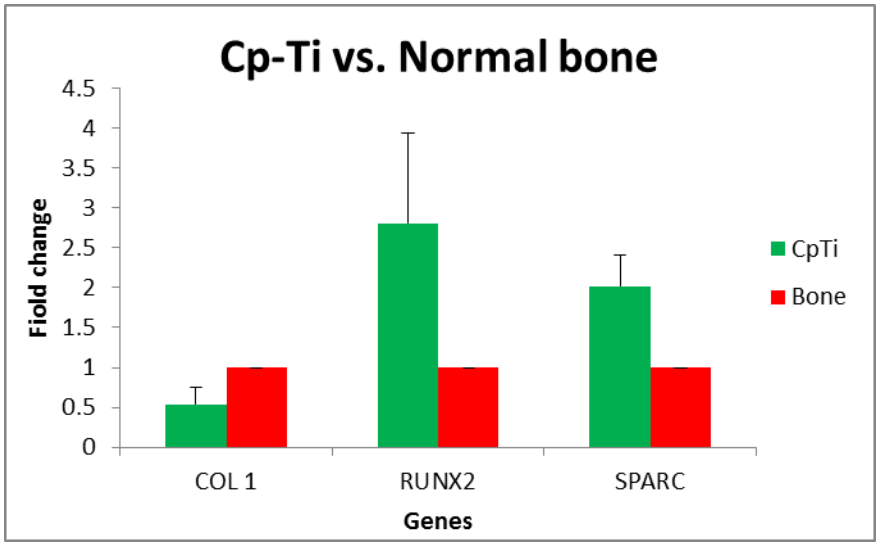


Fig: 8 Graphical representation of RT PCR data depicting the fold change in expression of bone-specific genes (values are represented in mean ±SD (n=5).P value >0.05. Abbreviations: Collagen 1 (COL 1); Runt-related transcription factor 2 (RUNX2,) Secreted protein acidic and cysteine-rich(SPARC)

The expression of RUNX2 and SPARC is up-regulated in Cp-Ti compared to normal bone.Further, the higher expression of RUNX2 demonstrates that Cp-Ti is involved in osseointegration mechanism during the process of bone healing.

3.1.4.1.3 Laser Manufactured Commercially Pure Titanium (LAM-Cp-Ti) vs. Commercially Pure Titanium (Cp-Ti)

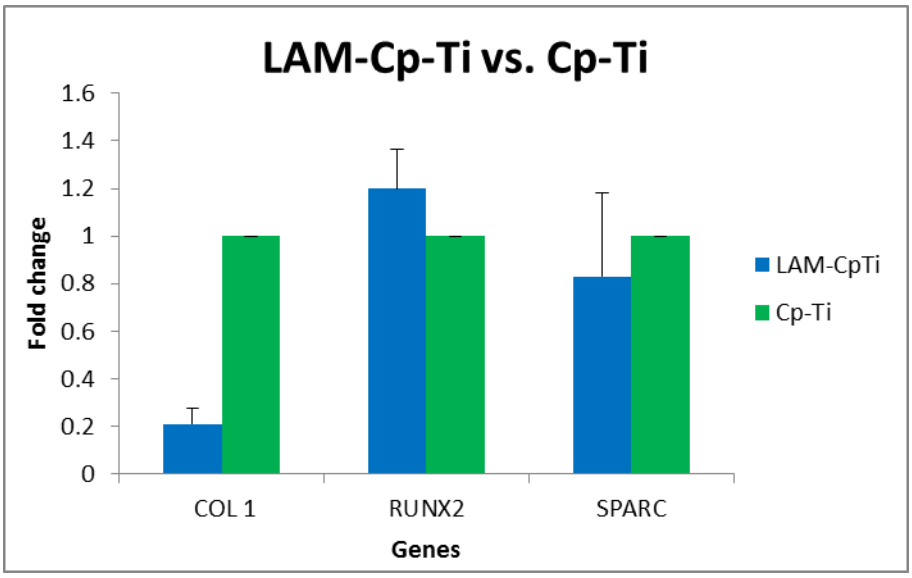


Fig: 9 Graphical representation of RT PCR data depicting the fold change in expression of bone-specific genes (values are represented in mean ±SD (n=5).P value >0.05. Abbreviations: Collagen 1 (COL 1), Runt-related transcription factor 2 (RUNX2) Secreted protein acidic and cysteine-rich (SPARC)

The expression of RUNX2 gene was found to be up-regulated in LAM-Cp-Ti compared to Cp-Ti. Whereas, expression of collagen 1 and SPARC was found to be down-regulated in LAM –Cp-Ti compared to Cp-Ti .There was no significant difference in terms of RUNX2 and SPARC expression between LAM-Cp-Ti and Cp-Ti. The higher expression of RUNX2 shows that LAM-Cp-Ti is capable of promoting osseointegration during the early stages of bone healing.

3.2 DISCUSSION

Osseointegration is essential for long-term success and inflammation free orthopedic implants. Surface properties of the implant have a direct role in osteogenesis at the bone-implant interface, influencing a series of coordinated events including protein adsorption, cell proliferation, and new bone tissue deposition. The advantage of porous materials is their ability to provide mechanical anchorage to surrounding bone tissues and greater contact area at the bone-implant interface. Three Dimensional Printing /Additive manufacturing techniques have been successfully used to fabricate implants with controlled and functionally graded porosity [Hollander et al., 2006; Traini et al., 2008; Ryan et al., 2008]. Laser additive manufacturing technique enables to control the porosity of each layer and is also possible to control the size, distribution, and interconnectivity of pores giving a controlled, open-pore network. LAM implants do not require post-fabrication process since they are not machined, no oils or contaminants are employed therefore no need of decontamination. Moreover, they do not need surface treatments, and this may further reduce the cost of production.

In this work, we studied the osseointegration ability of LAM-Cp-Ti implants by comparing with Cp-Ti implants after long-term implantation i.e. after six months in a rabbit model. We performed both histopathology and gene expression studies. Histopathological evaluation revealed the absence of necrosis, inflammation and intervening soft tissue between the implant- bone interface. Osseointegration was clearly evident from the new bone formation that was observed at the bone-implant interface .On microscopic evaluation, osteoblasts were observed in the marrow spaces. Thus from our study, we observed that both types of implants (LAM-Cp-Ti and Cp-Ti) promoted osseointegration. Many studies have been reported about osseointegration of additively manufactured orthopedic implants. All these studies showed promising results of osseointegration. Human teeth histologic studies have documented osseointegration after the placement of 3DP/AM titanium implants [Shibli et al., 2012]. Studies conducted by Witek and coworkers in 2012 using laser additive manufactured dental implants in dogs showed intimate contact between cortical and trabecular bone [Witek et al.,2012]. An

active zone of bone remodeling was observed between implant threads and adjacent compartments. Newly formed bone was seen to occupy all surface regions of the implants with marked signs of remodeling [Stubinger et al., 2013]. Modification of implant surfaces can improve the biological behavior around implants and can accelerate the process of osseointegration [Herro et al.2013]. A similar result was reported in a study conducted by Matena and co-workers in 2013, it was an in vitro study on laser additive manufactured titanium dental implants and they observed that osteoblasts were able to migrate and proliferate on a modified implant surface. [Matena et al., 2013] Recently, in 2017 Naiwen and coworkers conducted a six-month study of laser additive titanium dental implants in a diabetic pig model. Their results showed that the osteoblast cells grew and adopted on the surface of laser additive manufactured implants indicating osseointegration.

Bone healing follows a specific and complex signaling pathway and involves changes of the gene expression level. It is a highly coordinated process responsible for the repair of damaged bone and maintenance of mineral homeostasis. In addition to the bone cells (osteoclasts, osteoblasts, and osteocytes) that are necessary for bone remodeling, genetic mechanisms are also implicated in bone growth. They regulate the signaling molecules and proteins. The osteogenic genes investigated in our study were Collagen 1, SPARC and RUNX2. Collagen 1 gene has a specific role in mineralization process during new bone formation. SPARC is a bone-specific protein which selectively binds to collagen and encoded by SPARC gene. It is secreted by osteoblasts during bone formation. They have an affinity for collagen and calcium and also have a vital role in bone mineralization. RUNX2 gene is necessary for osteoblast differentiation [Schinke and Karsenty, 2008] and it can induce Alkaline Phosphatase (ALP) activity which helps in bone mineralization [Harada et al., 1999]. Moreover, it has been demonstrated that RUNX2 gene is involved during cell migration. It is postulated that up-regulation which may be involved in pre-osteoblasts attraction and their subsequent maturation and differentiation. In our study, we performed three types of comparison firstly, compared

gene expression of newly formed bone around LAM-Cp-Ti with normal cortical bone; secondly, the newly formed bone around Cp-Ti with normal cortical bone and thirdly, new bone formed around LAM-Cp-Ti with Cp-Ti.

When newly formed bone around LAM-Cp-Ti implant was compared with normal cortical bone up-regulation of collagen 1 and SPARC gene is seen in LAM-Cp-Ti when compared to normal bone. Collagen 1 and SPARC gene plays an important role in mineralization process during new bone formation. Its expression is increased in the areas of extracellular matrix and helps in collagen fibril assembly. SPARC gene binds to Extra Cellular Matrix (ECM) and therefore has the potential to contribute to the organization of matrix in connective tissue as well as basement membranes [Bradshaw et al., 2001]. In our study, the expression of SPARC gene has increased along with Collagen 1 gene and can be concluded that SPARC and Collagen 1 genes together are initiating active bone development and mineralization in normal skeletal tissue. RUNX2 gene is involved in osteoblast differentiation. Thus, it is clear that osteoblast differentiation has taken place already and mineralization phase of bone healing is taking place. Bone mineralization influences the mechanical strength of bone tissue [Boivin et al., 2009]

When the newly formed bone around Cp-Ti implant was compared with normal cortical bone, Collagen 1 gene shows a slight decrease in its expression compared to normal bone. The expression of genes RUNX2 and SPARC show increase in gene expression in the newly formed bone around Cp-Ti implant .This clearly shows that osteoblast differentiation and bone mineralization is continuing in the newly formed bone around the Cp-Ti implant.

In the final comparison, when newly formed bone around LAM-Cp-Ti is compared with new bone formed around Cp-Ti implant, Collagen 1 gene shows relatively low expression in LAM-Cp-Ti. RUNX2 gene shows relatively high expression in LAM-Cp-Ti. There was no significant difference in expression of SPARC in both LAM-Cp-Ti and Cp-Ti implants. It can be concluded that in LAM-Cp-Ti implant, osteoblast differentiation has taken place and bone development has progressed. The low expression

of Collagen 1 gene indicates mineralization phase is active. From this it can be concluded that LAM-Cp-Ti implant promotes osseointegration.

Recently, in 2017, Naiwen and co-workers conducted a six-month study using dental implants in a diabetic pig model. The study analyzed osteogenic-related gene expression and observed high expression levels of Collagen 1, and RUNX2, which demonstrates that osteoblasts can differentiate to bone tissue rapidly after attaching to the implant surface [Naiwen et al., 2017]. Rough surface could provide a better environment for fibrin clot stability and accelerate the progress of bone healing on the implant surface. Osseointegration at the bone-to-implant interface is influenced by several mechanisms including osteoblasts adhesion, proliferation, and bone deposition. All these mechanisms might be affected by different modifications of the implant surface.

CHAPTER 4

SUMMARY & CONCLUSION

4.1 SUMMARY

Present work was done to investigate the long-term osseointegration potential and biocompatibility of LAM-Cp-Ti implants obtained from Raja Ramanna Centre for Advanced Technology (RRCAT), Indore, Madhya Pradesh. The study was conducted on rabbit model (New Zealand White) after implantation in femur cortical defect .The long-term osseointegration and biocompatibility characteristics of LAM-Cp-Ti implants were compared to Cp-Ti implants. Histological analyses of the healing events associated with osseointegration in rabbit model have shown that osteogenesis occurs on both the implant surface. On microscopic evaluation, there was absence of necrosis, degeneration and intervening soft tissue between the implant-bone interfaces. The new bone formation was observed at the implant interface. Osteoblasts were also observed in the marrow spaces .All these observations clearly indicate new bone formation and osseointegration. Osteogenic gene expression study was conducted to analyze the expression of genes involved in bone formation. Genes examined were RUNX2, SPARC and Collagen 1. Osteogenic gene expression study shows positive results in the induction of genes by both types of implants. Three principal bone development periods – activation of osteogenic genes, extracellular matrix maturation, and mineralization is clearly evident by the expression of genes associated with each phase. These findings suggest that both Cp-Ti and LAM-Cp-Ti implants favor osseointegration.

4.2 CONCLUSION

The results of the present study proved good osseointegration and bone remodeling in both LAM-Cp-Ti and Cp-Ti implants after long-term implantation in a rabbit femur cortical defect model.

4.3 FUTURE PERSPECTIVES

- To elucidate the role of micro RNAs involved in osseointegration when implanted with LAM-Cp-Ti implants.
- To incorporate drugs on the surface of LAM-Cp-Ti implants.

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