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Effect of Nano Hydroxyapatite, Chitosan and Collagen Composite Coating on Commercially Pure Titanium Implants (Mechanical and Histological Study)

A thesis
Submitted to the Council of the College of Dentistry University of Baghdad, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Prosthodontics

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَعَلَّمَكَ مَا لَمْ تَكُن تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا

صدق الله العظيم

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Declaration

I certify that this thesis entitled "***Effect of Nano Hydroxyapatite, Chitosan and Collagen Composite Coating on Commercially Pure Titanium Implants (Mechanical and Histological Study)***" was prepared by ***Sabreen Waleed Ibrahim*** under my supervision at the College of Dentistry/University of Baghdad in partial fulfilment of the requirements for the Degree of Master Science in Prosthodontic Dentistry.

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Dedication

To My mother and My Husband……
Source of strength, love
and support.

My sisters and my lovely angels
Deema and Raghad….
Source of strength and hope …

And to everyone who support me
during this Study.

Sabreen

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Abstract

Background : In recent years Implantology evolved to occupy notable status in dentistry, dental implants provide comfort, stability , esthetic and nearly normal function so, it is regarded better choice for replacing missing teeth in comparison with removable prostheses for all indicated patients. Technologies for modifying surface characteristics or for coating were used to improve clinical success of dental implants.

Aim of study: to compare the effect of nano hydroxyapatite, chitosan and collagen composite coating, nano hydroxyapatite and chitosan composite coating with nano hydroxyapatite coating on commercially pure titanium implants by dip coating. Evaluation was made histologically and mechanically after 2 and 6weeks.

Materials and methods: Commercial pure titanium rod was machined into 54screws shaped implants. Implants were divided into 3 groups according to the types of coating used: 1st group (18 implants) coated by dip coating with nano hydroxyapatite (control), 2nd group include (18 implants) coated by dip coating with nano hydroxyapatite and chitosan composite, and 3rd group include (18 implants) were coated by dip coating with nano hydroxyapatite, chitosan and collagen composite.

Evaluation of surface chemical characteristics after coating (in vitro study) was done by X-ray diffraction (XRD) analysis, Fourier transfer infrared (FTIR) analysis and for evaluation of surface topography by scanning electron microscope (SEM) analysis and microscopical examination.

The tibiae of 18 white New Zealand rabbits were chosen as implantation sites.

Six implants for each type of coating were tested for torque removal test to measure bond strength between implant and bone for each period of healing, three screws for each type of coating were used for histological analysis after each healing period(2,6 weeks).

Results: The mean of torque removal for implants coated with nano hydroxyapatite, chitosan and collagen composite was more than implants coated with nano hydroxyapatite and chitosan composite and nano hydroxyapatite alone for both healing intervals, and implants coated with nano hydroxyapatite and chitosan composite record higher mean torque value than implants coated with nano hydroxyapatite alone for both healing intervals, mean value increasing with time for all coated groups of implants.

The histological examination illustrate a rapid bone formation for implants coated with nano hydroxyapatite ,chitosan and collagen composite more than implants coated with nano hydroxyapatite and chitosan composite and nano hydroxyapatite alone for both time intervals.

Conclusion: Implants coated with nanoHA, chitosan and collagen composite coating improved the mechanical and biological properties of bone after implantation, which was demonstrated by highest removal torque force, with acceleration in bone healing around dental implants as indicated by histological test at both time intervals.

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LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
CaP	Calcium phosphate
CoCrMo	Cobalt-chromium-molybdenum
Cp Ti	Commercially Pure Titanium
CoCr	Cobalt Chromium
CVD	Chemical vapor deposition
FTIR	Fourier Transform Infrared Spectroscopy
g/cm^3	Gram per cubic centimeter
GPa	Gigapascal
HA	Hydroxyapatite
H_2O_2	Hydrogen peroxide
JCPDS	Joint Committee For Powder Diffraction Standards
MPa	Megapascal
N.cm	Newton. Centimeter
Nb	Niobium
POWF	Pulse Oscillation Wave Form
PS	Plasma Spraying
SEM	Scanning electron microscope
Ti	Titanium
Ti 6- Al- 4 V	Titanium- aluminum-vanadium

Ti-6Al-7Nb	***Titanium- aluminum-niobium***
XRD	***X-ray diffraction***

Introduction

and

Aim of study

Introduction

In middle of last Century, Branemark reported formation of new bone on machined titanium implants. Titanium and its alloys are widely used in implantology due to their biocompatibility and lack of toxicity. Osseointegration affected by surface properties of implant, many studies reported that deposition of hydroxyapatite can improve the adhesion, proliferation, and mineralization of osteoblasts (***Goel et al., 2014).***

Increasing and accelerating the osseointegration of commercially pure titanium (Cp Ti) leads to reducing the non-functional time period of the implant, resulting in minimum discomfort to the patient, thus increasing the success rates ***(Sá et al. 2009)***

The implant surface characteristics can be categorized at different size levels. At a macroscopical level, the screws design, the thread shape and the pitch distance are basic parameters which affect the rate of osseointegration **(*Abuhussein et al 2010*)**, whereas at a microscopic level surface roughness appear to guide cell adhesion and differentiation of osteoprogenitor cells **(*Anselme and Bigerelle, 2000*).** At nanophase biomaterials cultured osteoblasts demonstrated good osteogenic behavior, involving adhesion, extracellular matrix (ECM) production and mineralization, in comparison to conventional materials ***(Elias et al., 2002).***

Although Titanium and its alloys are widely used in implant applications, but they may have some disadvantages, like low osteoinduction and low resistance to corrosion so coating titanium and its alloys with the bio ceramic material like hydroxyapatite is one of the ways to solve the problem (***Nie et al, 2000***).

Dip coating can produce thin homogenous coating, providing better control of the chemical composition and macrostructure of the coating as well as

improving the biological activity of the titanium implants which enhance bone formation (***Anil et al .2011***)

Similarity of the physical properties of the polymeric materials with those of the organic phase present in bone tissues is the reason of polymeric material being widely used in implantology field, these properties enhancing formation of new bone structures and supporting the fastest osseointegration (***Carlos et al., 2010)***

Chitosan has the capability to be used as a biocompatible, bioactive coating for implant devices because it has ability to stimulate osteoblast cells (***Khora and Limb, 2003)***

While Collagen is a natural polymer, is extensively being applied in tissue engineering and repair. It is has excellent biocompatible properties and allows good adhesion and regeneration of cells (***Alexander et al., 2012).***

Aims of the study

Comparison between nano Hydroxyapatite, nano Hydroxyapatite and chitosan composite and nano Hydroxyapatite, chitosan and collagen composite coating on commercially pure titanium screws by dip coating in:

1. Removal torque value of the bond strength between coated implants and bone after different periods of implantation (2, 6) weeks after implantation in rabbit tibia.
2. Evaluation the biocompatibility of coating materials on bone healing around Ti implants histologically after 2 and 6 weeks intervals after implantation in rabbit tibia.

Chapter One

Review of Literurature

1.1 Bone

Bone is a highly specialized connective tissue consist of bone matrix, osteogenic cells (include osteoblasts, osteocytes and osteoclasts), and vasculature. Bone matrix consists of both inorganic and organic phases. Calcium phosphate crystals are the main components of the inorganic part ***(Junqueira and Carneiro, 2005).***

Bone tissue can be divided into organic and inorganic components, which corresponds to 33% and 67% of the weight of bone, respectively with water content

The bone organic matrix is formed mainly by collagen type I and trace amounts of type V and XII (90%, approximately), the remaining 10% of the organic matrix is formed by a variety of non- collagenous proteins (include osteocalcin, osteonectin, bone sialoproteins, bone phosphoproteins and proteoglycans) that have different functions on the regulation of bone mineralization, organization of the matrix and activity of bone cells. The inorganic matrix serves as an ion reservoir and gives bone most of its stiffness and strength ***(Bilezikian et al, 2002).***

To appreciate biologic mechanism of bone healing and adaptation must have knowledge of bone types:

1.1.1 Macroscopical structure of Bone

In the macroscopical level there are two types of bone: cortical and cancellous. Matrix composition and structure of Cortical is similar to cancellous bone ,but the mass of the cancellous bone matrix per unit of volume is lower, with approximately 50-90% of porosity compared to 10%porosity found in the cortical bone. This difference in tissue arrangement provides increased resistance to torsion and bending to the cortical compared to the cancellous bone ***(Bilezikian et al, 2002).***

1.1.2 Microscopical structure of Bone

In the microscopical level, cortical and cancellous bone may consist of woven or lamellar bone. The surfaces of bone are covered externally with connective tissue sheets called periosteum and internally with endosteum. The periosteum contributes an important part of the blood supply to the bone and exhibits mesenchymal cells that may differentiate and form osteoblasts and osteocytes (***Bilezikian et al., 2002)***

I / woven bone:

Woven bone is formed when the deposition of collagen fibers occurred with no particular configuration. It has a lower mineral density (darker on X-rays) and more osteocytes than mature bone and it is the first bone form in embryos and in bone repair. Woven bone is weak, disorganized, and poorly mineralized ***(Junqueira and Carneiro, 2005).***

II /Lamellar bone:

It is a strong, highly organized, well mineralized tissue, make up more than 99% of adult human skeleton. Lamellar bone exists in two macroscopically forms: trabecular (cancellous) and cortical (compact). when new lamellar bone is formed, a portion of the mineral component (hydroxyapatite crystals) is deposited by osteoblasts during primary mineralization. Secondary mineralization which completes the mineral component, requires many months, within physiological limits, the strength of bone directly related to mineral content ***(Davies and Park,2003).***

III /Composite bone:

An osseous tissue formed by the deposition of lamellar bone within a woven bone lattice, by cancellous compaction process to produce strong bone in a short time. It is the main osseous tissue for stabilization during early stage of healing. Composite bone is high quality, it remodeled in to secondary osteons ***(Roberts et al,1989) .***

1.2 Dental implant

Dental implant is a prosthetic device of alloplastic material(s) inserted into the oral tissues beneath the mucosal and/or periosteal layer, and on/or within the bone to provide retention and support for a fixed or removable prosthesis (***Glossary of prosthodontic terms, 2005).***

Over the world, implantology has developed into the mainstream of restorative practices. Some of the advantages of dental implant for the edentulous patient are preservation of bone after tooth loss to enhance or maintain facial esthetics with improved retention, function as well as performance of removable restorations.. No longer are implants considered just when classical restorations cannot be fabricated ***(Misch, 2001*)*.***

1.2.1Types of implants

Dental implants generally can be classified into three main groups ***(Weiss and Weis, 2001).***

I. Endosseous implants:

These are implants that are surgically inserted within the jaw bone. Endosteal dental implant is a device implanted into the alveolar and/or basal bone of the mandible or maxilla and transecting just one cortical plate **.**

II. Subperiosteal implants:

These are implants not penetrate into the jaw, placed on top of the jaw bone underneath gum tissue .

III. Transosseous implants:

Bicortical implants because they are surgically placed within the jaw bone by penetrating the entire jaw so that they emerge from opposite site to entry site, commonly used at the bottom of the chin.

1.3 Osseointegration

Branemark defined **osseointegration** as "a direct structural and functional connection between the living bone and the surface of implant without interposition of non-bone tissue, that means the non-vital components can be inserted into living bone and that this implantation can stay under typical conditions of loading" **(Branemark, 2001).**

Albrektsson & Johansson, (2001) defined osseointegration clinically as asymptomatic firm fixetion of the implant is accomplished and preserved in bone during functional loading

Actually, bone must integrate with the dental implant material rather than respond to the material as a foreign body **(*John et al, 2006).***

After a defined period of healing, an implant is supposed to be osseointegrated where there should be no movement between the implant and tissue bed under typical conditions of loading ***(Wenz et al., 2008).***

Osseointegration determine the stability of implant, and is considered a necessary condition for implant loading and long-term clinical accomplishment of dental implants **(*Sul et al, 2005)***

During the past two decades, many approaches were interested on finding methods to accelerate and improve osseointegration to withstand occlusal forces at an early period, because the main objective of implant dentistry was increasing the local quality and quantity of the host tissue for optimal osseointegration ***(Morton et al., 2010).***

1.3.1 Biology of osseointegration:

Forming of new bone on the surface of implant is by serial of cellular and extracellular biological processes at the bone-implant interface, cellular and extracellular biological processes regulated by growth and differentiation factors, released form activated blood cells at the surgical site ***(Fini et al, 2004)..***

After placing of implants blood coagulation and hematoma formed, initial interactions of blood cells with the implant influence clot formation. Platelets undergo morphological and biochemical changes as a response to the foreign surface including adhesion, spreading, aggregation, and intracellular biochemical changes such as induction of phosphotyrosine, intracellular calcium increase, and hydrolysis of phosphor lipids. The formed fibrin matrix acts as a scaffold for the migration of osteogenic cells and eventual differentiation of these cells in the healing compartment ***(Meyer et al., 2004)***. thrombin and growth factors released from leukocytes and platelets in the hematoma, these factors attract many cell types which have an important role in bone healing and formation of procallus, which consist of fibroblasts, fibrous tissue and phagocytes. The procallus becomes dense connective tissue and mesenchymal cells differentiate into osteoblast that appears on the fixture surface The difference between healing of bone tissue and soft tissue that healing of bone tissue does not leave scar **(*Stanford and Schneider, 2004*)**.

Alkaline phosphatase, Bone matrix proteins, and osteocalcin are important signals to osteogenic differentiation and bone tissue formation, had appeared at higher number on rougher titanium and titanium alloy surfaces of implants (***Davies, 1998)***

Osteoblasts and mesenchymal cells seem to migrate and attach to the implant surface from day one after implantation, depositing bone-related proteins and creating a non-collagenous matrix layer on the implant surface that regulates cell adhesion and binding of minerals. This matrix is an early-formed calcified a fibrillar layer on the implant surface, involving poorly mineralized osteoid similar to the bone cement lines that forms a continuous, 0.5 mm thick layer that is rich in calcium, phosphorus, osteopontin and bone sialoprotein ***(Meyer et al., 2004)***.

Murai et al. in 1996 were the first to report a 20-50 mm thin layer of flat osteoblast-like cells, calcified collagen fibrils and a slight mineralized area at a titanium implant-bone interface.

The newly formed bone was laid down on the reabsorbed surface of the old bone after osteoclastic activity. This suggested that the implant surface is positively recognizable from the osteogenic cells. Cement lines of poorly mineralized osteoid demarcated the area where bone reabsorption was completed and bone formation initiated. A few days after implantation, even osteoblasts in direct contact with the implant surface began to deposit collagen matrix directly on the early formed cement line/lamina limitans layer on the implant surface(***Fini et al, 2004)***.

Rapid woven bone formation (10 to 14 days after surgery) occurs on implants to restore continuity, even though its mechanical properties is lower compared to lamellar bone based on the random orientation of its collagen fibers. Woven and trabecular bone fill the initial gap at the implant-bone interface. Arranged in a three-dimensional regular network, it offers a high resistance to early implant loading. Its physical architecture including arches and bridges offers a biological scaffold for cell attachment and bone deposition that is biological fixation (***Franchi et al. 2005)***

Then, woven bone is gradually remodeled and substituted by lamellar bone that may reach a high degree of mineralization. At three months post-implantation, a mixed bone texture of woven and lamellar matrix can be seen around implants ***(Rigo et al. 2004)*** .

1.3.2 Factors which determine the success of osseointegration

Lee et al, in 2010 stated that success of osseointegration affected by six important factors**,** these are:

1. Implant material biocompatibility
2. Implant design and shape
3. Status of the bone

4. Surgical technique
5. Implant loading conditions
6. Surface conditions

1.3.2.1 Implant material biocompatibility:

Corrosion resistance, non-toxicity, modulus of elasticity, and fatigue strength are properties of biomaterials which can affect the selection of the right biomaterials for a specific biomedical application. The implant Material surface plays an important role in the response of the biological environment to artificial medical devices ***(Liu et al., 2004)***.

Biocompatibility of material, chemistry and microtopography of the implant surface are very important properties because these properties participate to its osteoconductivity, if the ability of osteoconductivity is improved, implants osseointegration improved, resulting in success of dental implant. **(*Liu et al, 2007).***

Biocompatibility is one of the most important factors,it means the ability of the implant to perform a suitable host response in its specific application ***(Weiss and Weis, 2001).*** This means that implant materials that come into contact with the host tissues does not suffer from any toxic, irritating, inflammatory, allergic, mutagenic or carcinogenic condition **(*Vahey et al, 1995*).**

The ideal dental implant material should be ***(Edgerton and Levine, 1993; LeGeros and Craig, 1993):***

1- Biocompatible.

2- Sufficient rigidity for prosthetic function.

3- Adaptable to both bone and gingiva surrounding the implant.

4- Able to dissipate forces resulting from occlusal load on the prostheses supported by the implant to the underlying bone.

In fact, all these characteristics not meet in one dental implant material, so, various coating technologies were developed to enhance implant biocompatibility at host-implant interfaces ***(Aspenberg et al., 1996)***.

Using of an implant material which is not biocompatible with surrounding tissue may result in implant failure and adverse reactions of tissue bed ***(Santavirta et al., 1999)***.

The biocompatible materials can be classified depended on their effects on body tissues to:

I. Bio tolerant material :

Stainless steel, Bone cement, and Cr-Co alloy are examples of biotolerant material, they have ability of a distant osteogenesis; bone will form but not in contact with the host bone. The implant retention is based on the principle of interlocking exclusively by mechanical mean**…**

II. Bioinert material:

Example: carbon, alumina and titanium, it showed contact osteogenesis; direct contact of the adjacent bone to the implanted material. The implant is only mechanically retained.

III. Bioactive material :

Example: glass-ceramics, tricalcium phosphates and hydroxyapatite .It showed bonding osteogenesis; direct chemical bond between implant and bone. The implant retained mechanically and chemically.

Gonzalez et al in 2003 classified the biomaterials into metals, ceramics, composites, and polymers.

1.3.2.1.1 *Metals*:

Biomechanical properties; previous experience with processing, treating, machining, and finishing; and suitability for common sterilization procedures these are factors must be considered during selection of metallic materials for implants. Fabrication of dental implants from many metals and metal alloys

may result in adverse tissue reactions, and low success rates. Different metals and alloys (gold, stainless steel, and cobalt-chromium) are not utilized nowadays as part of dental implant, while titanium (Ti) and its alloys (mainly Ti-6Al-4V) have become broadly utilized for dental implants fabrication **(*Nikitas et al., 2000)***.

1. Titanium:

Over the past three decades, Ti has been widely used in dentistry. Cotton J. in 1947 was introduced Ti and its alloys as implants with medical applications…

According to the American Society of Testing Materials (ASTM), Cp Ti is available in four different grades (Grade I-IV) which vary according to the oxygen content ranging from0.18 to 0.40 weight percent and iron content ranging from0.20 to 0.50weight percent; traces of other elements like nitrogen; carbon, hydrogen, and iron have also been added improvement of the mechanical and physicochemical properties. Iron is added for corrosion resistance and aluminum is added for increased strength and decreased density, while vanadium acts as an aluminum scavenger to prevent corrosion ***(Meffert etal., 1992; Williams, 1981)***.

Table 1.1 summarized some basic physical properties of commercially pure titanium (Cp Ti) **(*Liu et al., 2004)***.

Property	**value**
Atomic number	22
Atomic weight (g/mol)	47.90
Density (g cm-^{3})	4. 54
Coefficient of thermal expansion,α,at 20 ^{o}C (K-1)	8.4×10-6
Melting temperature (^{o}C)	1668

Boiling temperature (estimated) (^{o}C)	3260
Transformation temperature (^{o}C)	882.5
Modulus of elasticity,α, (Gpa)	105
Yield strength,α,(Mpa)	692
Ultimate strength, α, ,(Mpa)	785

Ti density is 4.54 g/cm^3 less than that of other metals used in dentistry, like gold (19.3g/cm^3) or CoCr Mo alloy (8.5 g/cm^3). In its unalloyed condition Ti is strong like steel, but it is lighter (density of stainless steel is 7.9 g/cm^3). Its melting point is 1668°C, and its other thermal properties (like thermal conductivity) are similar to those of the dental tissues..

Freese et al., (2001), stated that Ti cannot use in major loadbearing applications because shear strength of Ti is too low as well as it has low wear and abrasion resistance

Ductility and strength of Ti is greatly affected by amount of Oxygen. ***(Park and Kim, 2000; O'Brien, 2002)***.

Niinomi, in ***(2011)*** stated that Cp Ti alloys can be categorized as α-, (α + β)-, and β-type alloys. Generally, α-phase titanium is stronger but less ductile and β-phase titanium is more ductile, while the mechanical properties of (α + β)-type titanium are in between both..

2. Ti alloys:

In the mid-1980s the Ti-6Al-7Nb alloy was introduced as alternative to Ti-6Al-4V, because niobium is more biocompatible and cheaper than vanadium**..** Ti-6Al-7Nb showed the best workability and mechanical properties. **(*Semlitsch et al, 1995)***

On the other hand the Ti6Al4V alloy has become desired due its chemical resistance, mechanical strength, lightness, low toxicity and excellent

biocompatibility.. It was the first Ti alloy to be registered as an implant material **(*Kitamura et al 2002).***

1.3.2.1.2 Ceramics:

Ceramics can be defined as hard inorganic, nonmetallic materials contain compounds of oxygen with one or more metallic or semi-metallic elements like aluminum, calcium, lithium, magnesium, phosphorus, potassium, silicon, sodium, zirconium and titanium, ceramic materials is brittle, corrosion resistant, high compressive strengths, and withstand high temperature. Because of crystalline structures of ceramic is similar to bone so, the physical properties of ceramic similar to bone (***Isa and Hobkirk, 2000).***

Ceramics when used as implants or as coatings to metal restorations, they can initiate bone formation, improve tissue growth as well as providing protection from the immune system **(*Thamaraiselvi and Rajeswari, 2005)***

Ceramic material used in the construction of dental implants can be classified into:

1. Non absorbable (Bioinert ceramics): which include carbons, alumina, silicon nitrides, polymers and zirconia.
2. Bioactive or surface reactive (semi-inert): include certain glass ceramics and dense hydroxyapatites.
3. Biodegradable or resorbable (non-inert): include calcium phosphates and calcium aluminates ***(Park and Lakes, 1992)***.

- **Calcium phosphate**

Calcium phosphate (CaP) is the most important type of inorganic materials used to alter implant surfaces. There are numerous types of (CaP) compounds like Hydroxyapatite (HA, Ca_{10} $(PO_4)_6$ OH_2) and tricalcium phosphate (TCP, $(Ca_3\ PO_4)_2$, etc. **(*Kumta et al., 2005).***

- **Hydroxyapatite (HA)**

HA [$Ca_{10}(PO_1)_6OH_2$]. It is an inorganic material, inert bioactive ceramics and chemical structure of HA is similar to the bone **(Rohanizadeh *et al.,* 2005).** HA has ability to enhance the attachment and growth of human osteoblast-like cells, so it can improve new bone formation .HA has the ability to bond directly to bone tissues without fibrous encapsulation and tissue infection, in addition to that, HA coating can improve the success rate of implants, because HA ceramics can support attachment, proliferation, and differentiation of mesenchymal stem cell (MSC)**.**

HA being used in bone regeneration, as a substitute of bone and teeth, coating for metal implants and maxillofacial reconstruction because it is a biocompatible, bioactive, non-inflammatory, non-toxic, osteoconductive and non-immunogenic material as stated by **(*Zhao et al., 2006*)**.

1.3.2.1.3 *Polymers* :

Polymeric materials are limited to be used as shock-absorbing components of supra structures supported by implants **(*Chapman and Kirsch, 1990*)**.

Various polymers used as dental implant materials, like ultrahigh molecular weight polyurethane, polyamide fibers, polymethylmethacrylate resin, polytetrafluoroethylene, and polyurethane **(*Lemons, 1990; Carvalho,1997*)..** The flexibility of polymers would simulate the micromovement of the periodontal ligament **(*Meijer et al.,1995*)**. The flexible implants compared to rigid implants has capacity to transfer stress more favorable to bone **(*Meijer al.,1997*)**..

1.3.2.2 Implant design :

To improve the initial implant stability, the implant design must be adapted to different bone situation, Implant stability in low density bone can be affected by the design of implant, whereas bone with high density the effect of design is less **(*O'Sullivan et al, 2000;Friberg, 2002)***.

For most applications parallel implants is not appropriate, tapered implants introduced to improve aesthetics and aid placement of implant between adjacent natural teeth **(*Shapoff, 2002).*** Labial bone perforation especially in thin alveolar ridges may occur when using implants with cylindrical wide body **(*Garber et al., 2001*).**

Inadequate implant design can be compensated when the quality and quantity of bone are optimal **(*Chong et al., 2009)***.

Dental implants must be designed to increase surface area of implant and to maximize favorable stresses and to minimize unfavorable stresses applied on the implant, this greatly affects the osseointegration **(*Abuhussein, 2010*).**

1.3.2.2.1 Implant length :

Mechanical analysis showed that increasing success rate of implants with increasing implant length but with limited degree **(*Lum, 1999).***

Douglass and Merrin(2002) Suggested that there is no relation between implant length and implant mobility**..** Reduced stress at the cancellous peri-implant area may occur with increase in the implant length ***(Baggi et al, 2008).***

1.3.2.2.2 Implant diameter:

Theoretically using of wide implants enhance distribution of stress in the surrounding bone, permits engagement of a greater amount of bone , and permits for the application of higher torque in the placement of prosthetic component **(*Ivanoff et al, 1997*). .**But, using of wide implants is restricted by the width of residual ridge and esthetic requirements **(*Ettinger et al, 1993).***

Implants with narrow-diameter have been introduced for residual ridges with limited interdental width **(*Vigolo and Givani, 2000 ; Minsk, 2001).***

But, the drawback of narrow diameter implants is the reduction in resistance to occlusal loading **(*Himmlova et al, 2004).***

***Baggi et al., (2008*)** suggested that Implant diameter assume more important as a design parameter than implant length to prevent bone overload risk.

1.3.2.2.3 Implant shape:

Many implants designs are available as solid or hollow screw or cylinders . To increase self-tapping in screw design and reduce heat generation, many modifications can be made to the crystal and apical portion of the implant. ***Kan et al, (2002)*** stated that screw shaped implants gives the greatest retention, immediately following implant insertion. Increased pitch and increased depth between individual threads of dental implants allowing for enhanced contact area between bone and implant and can modify the biomechanical properties.. Implant threads should be designed to increase the favorable stresses while reduce the amount of adverse stresses to the bone implant contact and implant threads should permit good stability and more implant surface contact area (***Abu Hussein et al., 2010*).**

1.3.2.3 Status of bone :

It is very important to assure the absence of local or systemic bone disease because the biologic host tissue can determine the success of the implanted materials **(*Khan et al, 1999)***

Length, width, and depth are dimensions of bone, available bone dimension and the dimensions of dental implant must be estimated. If quantity of bone is inadequate so must use smaller implant and this will increase the failure rate ***(Weiss and Weis, 2001*).**

The term bone quality is frequently used in dental implant treatment and in description of implant success and failure, where status of bone is very important in primary stability **(*Lindh et al. 2004.)***

Bone quality and bone density (Bone Mineral Density, BMD) are not equivalent. Bone quality involves skeletal size, and matrix properties. Bone quality is not only an issue of mineral composition, but also includes

structure. The success of dental implants greatly depends on the quality and quantity of bone at the implantation site; different healing periods may occur in different types of bone ***(Drage et al., 2007; Lindh et al., 2004)***.

The mechanical properties at the implant-bone interface are improved by bone quality. Implant in high density bone showed to have less micro movement **(*Misch, 2008*).**

During designing implant treatment, it is very important to assess the quantity and quality of bone of the jaws, because the success rate gained with dental implants greatly depends on the volume and quality of the surrounding bone **(*Ribeiro et al, 2010)***

According to the proportion and structure of compact and trabecular bone tissue quality of bone can be classified into four groups **(*Ribeiro et al, 2010*)**

Type I: homogeneous cortical bone.

Type II: thick cortical bone with marrow cavity.

Type III: thin cortical bone with dense trabecular bone of good strength

Type IV: very thin cortical bone with low density trabecular bone of poor strength.

1.3.2.4 Surgical technique:

Necrosis happens when the temperature more than 47 º C for 1 min or 40 º C for 7 min, so care must be taken to avoid thermal bone damage during the drilling **(*Friberg et al., 2001)*.**

Atraumatic surgical technique is very important to reduce mechanical and thermal damage and aid in integration of the implant **(*Sharawy et al, 2002)*.**

Mechanical damage to the host bone and increases the temperature of the bone directly adjacent to the implant surface can occur during drilling of host bone resulting in destructive effect on the bone tissue around the dental implant **(*Anitua et al., 2007)*.**

keeping the temperature below 47 ºC can achieved by using external irrigation at room temperature and graduated set of sharp drills work intermittently at low speeds **(*Sener et al, 2009*)..**

Insertion torque should be about (15-30rpm), because high level of insertion torque may result in stress concentration around the threads of the screw-type implant, and this may result in bone resorption **(*Ashly et al, 2003*).**

1.3.2.5 Implant loading condition :

About 3 to 6 months of unloading healing period is considered critical. This unloading period permits for the differentiation of primitive mesenchymal cells to bone forming cells and for initial callus formation **(*Cochran et al, 2004)*.**

Studies reported that there are three options for implant loading:

I. ***Immediate / direct loading***: the temporary dental prosthesis is connected to the implant and allow loading within the first week after insertion .
II. ***Early loading***: in this loading condition the provisional dental prosthesis connected to dental implant within few days or weeks after the implant placement.
III. ***Delayed loading /2 stage technique***: the temporary dental prosthesis is connected and allow loading at a second surgical visit after a complete healing period of 3 months in the mandible and 6 months in the maxilla **(Ostman, 2008)**.

1.3.2.6 Implant surface condition:

Implant surface modifications aimed to improve osseointegration, and accelerate bone formation and consequently improving stability of implant during the healing process **(*Li J et al., 2007)***.

***Ruggero et al., (2012*)** stated that Implant surface modification involve coating and topographical modifications. The goal of topographical

modifications of implant surface was to enhance the roughness of implant surfaces, thus increasing the surface area of implants, by increasing surface area to cell attachment and the biomechanical engagement between surrounding bone and the implant are increased. Fibroblasts and epithelial cells attach strongly on smooth surfaces and the ability of osteoblast to adhere and proliferate and collagen production is higher on surface with moderate roughness.

According to the formation mechanism of the modified layer on the implant surface, Surface modification methods of implants, can be classified into the mechanical, chemical and physical methods (***liu X,et al.,2004)***

I. Mechanical methods:

The goal of mechanical modification is to gain particular surface topographies, to clean or roughen surfaces, resulting in increasing surface area, enhancing osseointegration; these methods involve physical forces applied on the implant surface to modify the surface characteristics (***Gongadze et al., 2011)***

1. Machined surfaces

Machined surface is a description of a turned, milled or sometimes a polished surface **(*Wennerberg et al, 2000*)**.

Modification in the surface topography have been used to influence the cell and tissue responses to the implants **(*Gongadze et al., 2011)***

2 .shot blasted surface

It is simple process, the roughness of surface that gained by this technique is typically sharp and irregular; the medium grit particles are 250-500 μm while the large grit sand blasting particles are about 0.25-0.5mm in size **(*Orsini et al, 2000)***.

This technique involve using a high speed sputtering of blasting grits at high pressure.Al2O3 is the most common material used for this purpose (***Li et al, 2001*)**.

II. Chemical methods:

These methods provide metallic surface with bioactive surface characteristics, The goal of using chemical methods is to enhance biocompatibility, bioactivity and improve corrosion resistance and removal of contamination (***liu X et al.,2004)***

1. Chemically etched surface

Acid etch has important role in production of nano roughness, which affect protein adhesion which takes place immediately following the implantation **(*Wennerberg et al, 2010)***.

Mixing of strong acids is efficient in gaining a thin grid of nanopits on a titanium surface about 75 nm in diameter. The titanium sample could be etched with a solution of strong acids, e.g., H_2SO_4 and H_2O_2, at a specific temperature and for a constant duration. Then etching is stopped by adding distilled water **(*Variola et al,2011).***

2. Hydrogen peroxide treatment

Chemical dissolution and oxidation of titanium surface happened and producing the of a layer of amorphous titania gel on the Ti surface as result of reaction between H_2O_2 and titanium ***(Yamamoto et al., 2005; Mediaswanti et al.,2013)***.

Thickness of titania gel layer formed on the surface depend on time of treatment, A shorter chemical treatment time result in thinner gel layer with higher porosity typically submicrometer in size(***Wang etal.,2002)***.

3. sol-gel deposition

The sol-gel deposition technique depends on colloidal suspensions of solid particles in a liquid solution (sol). The sol-gel method include multiple ways like spraying, dip coating, and spin coating. The gel form of coating is put

on the surface of object and only the precursor materials are left as thin film after drying (***paital and Dahotre,2009).***

The advantages of the sol-gel method include: ability to give fine and even grained structures, using various chemical compounds with their ease of application to complex shapes and high homogeneity because mixing done on the molecular level. Sol –gel method is one of the commonly used technique in deposition of thin coatings on implant surface (***Jamil, 2011*)**.

Dip coating technique

Dip coating method can be defined as process of coating when the object is placed in a liquid and then picked with well –defined speed under controlled temperature and atmospheric conditions . Dip coating is a technique of obtaining thin films of coating for research approaches. The mechanical properties, microhardness and abrasion resistance of coating-object system increased as elevating the firing temperature and viscosity of coating solution. Dip coating easy to perform, low cost, and accommodate shape variation ***(Hanyaloglu, 2008).***

Dip coating methods have been also used for coating glass and lenses because it can well adapt curved surfaces (***Ivankoviaei,2005)***.

Final film thickness of coating layer influenced by viscosity and dipping speed. The higher viscosity of the solution, the more of it will adhere to the substrate surface resulting in a thicker film, on the other hand increasing the withdrawal speed, the thicker the coating will result (***Mellor, 2001).***

Stages of dip coating

Basically this process may be separated into three important technical stages:

1. Immersion and dwell time: The substrate is immersed into the precursor solution at a constant speed followed by a certain dwell time in order to leave sufficient interaction time of the substrate with the coating solution for complete wetting.

2. Deposition & Drainage: By pulling the substrate upward at a constant speed a thin layer of precursor solution is entrained, i.e. film deposition. Excess liquid will drain from the surface.
3. Evaporation: The solvent evaporates from the fluid, leaving deposited thin film, which can be promoted by heated drying. Subsequently the coating may be subjected to further heat treatment in order to burn out residual organics and induce crystallization of the functional oxides. (***Ivankoviaei,2005 and Brinker2013) .***

4. Biochemical methods

Biochemical methods depend on the deposition of foreign chemical substances on the implant surface by biomimetic precipitation of calcium phosphate through immersion in simulated body fluid ***(Morra, 2006 and Mas-Moruna et al.,*** .**2013**)

5. Electrochemical methods

Electrochemical technique involves the connecting of the metallic substrate to the anode of an electrical circuit and immersing the entire device into an electrolyte solution containing ionic substances or oxidants. This methodology can result in deposition of some ions on the material surface, and involves the possibility of modifying the surface finish ***(Liu X et al., 2004).***

Micro-Arc Oxidation (MAO) is another electrochemical method which has been widely used for deposition of ceramic coatings on the surface of metals, this method can result in good adhesion, high strength and wear resistance could be also attained (***Krzakala et al., 2013).***

6. Chemical vapor deposition

Chemical vapor deposition is a method encompassing chemical reactions between chemicals in the gas phase and the material surface leading to the

deposition of a non-volatile compound on the substrate. CVD is widely used in the industry to form organic and inorganic films on metals ***(Liu X et al., 2004).***

III. Physical methods:

Physical methods result in the formation of modified layer, films or coatings on titanium and its alloys, mainly caused by either thermal or kinetic, or electrical energy.

1. Plasma-spraying method

The plasma –spraying (PS)technique results in deposition of relatively thick coating layer (>30μm), this involves the introduction of precursor like HA(feedstock) into hot plasma jet generated by plasma torch(**Gross et al,2009)**, at atmospheric pressure (Atmospheric Plasma Spraying ,APS),under vacuum (Vacuum Plasma Spraying ,VPS)or under reduced pressure (Low Pressure Plasma Spraying, LPS), because of partial or complete melting of powder particle an adherent coating is formed (***Huang et al.,2010).***

2. Ion implantation

This method include modifying surface of implant to depth of about 10 nm by energetic bombardment in order to enhance mechanical quality **(*Lee et al, 2001*).**

Nitrogen ion implantation on Ti-6Al-4V and Ti-6Al-7Nb alloys have been reveal to improve the passivity and decrease the corrosion kinetics of the surface with increasing tendency for passivation (***Thair et al.,2003*).**

3. Glow discharge plasma treatment

Glow discharge plasma is a method involving the use of a low-temperature with low-pressure gas in which ionization is controlled by energetic electrons. Glow discharge plasma treatment has been used for

cleaning as well as surface processing in the microelectronics industry and biomaterials research ***(Liu X et al., 2004).***

4. Physical vapor deposition

Physical vapor deposition process involve evaporation of the target materials in vacuum, to form atoms, molecules or ions then transported to the substrate surface, on which some reactions with the materials surface take place leading to film growth. ***(Liu X et al., 2004).***

5. Pulsed-laser deposition method

Pulsed-laser deposition method was used to produce a thin coating layer (0.05-3μm) on metallic substrate. These layers are adherent to enhance osseointegration. This method was directed towards the creation of higher crystalline films at low substrate temperature **(*Blind et al, 2005).***

1.4 *Methods used to evaluate implant stability*:

Implant stability it is important factor that affect the time of implant loading and the success of treatment **(*Testori et al., 2002)***.

In human is difficult to assess osseointegration of dental implants because most of techniques are designed for retrieved implants so, could not be stay functionally stable in patients. Therefore the methods of evaluation had been categorized into invasive methods and non-invasive methods **(*Palmquist, 2008)*** and ***(Zix et al 2008).***

A. Non-invasive method include (Radiological test, Percussion test, Pulsed oscillation wave form (POWF), Implant hammer method (IHM), Resonance Frequency Analysis (RFA), Dental Mobility Checker (DMC), Periotest, Finit element analysis).

B. Invasive method include (Cutting torque resistance analysis (CTRA); Removal torque test; Push-out/pull-out test; Histomorphometric analysis; Insertion Torque Analysis)

A. Non-invasive method:

1. Radiographic evaluation

***Hermann* et al, (2001)** stated that evaluation of the quantity and quality of bone at the site of an implant can be done by radiography, it can also provide indirect estimation of the density. Radiographic evaluation is more commonly used and more efficient during dental implants treatment. Radiographic analysis used to assess orientation of dental implant parallel to the long axis of the surrounding teeth. Bitewing radiographs are used to measure crestal bone level which is essential for implant success. There is possibility of distortion especially when the central x-ray tube is not positioned parallel to the structures of interest**. (Misch, 2005)**.

They can be used to assess changing in bone tissue mineral only when the mineralization decrease over 40% **(Goodson et al, 1984, Jong-Chul Park et al., 2011)**.

With the development of radiographic technology, Computed tomography (CT), as well as cone-beam computed tomography (CBCT) is increasingly considered essential for optimal implant placement. ***(Benson & Shetty, 2009).***

2. Pulsed Oscillation Waveform

The frequency and number of implant vibrations generated by a little pulsed force is basis of this technique. Multi-frequency pulsed force of 1 kHz is directed to the implant by gently tapping it with two small needles attached to piezoelectric structures(***Atsumi et al.*, 2007)**.

3. Impact Hammer Method

In this method the sound produced from hammer- object contact is recorded by Fast Fourier transform (FFT) and many devices have been introduced for detection the osseointegration, such as a microphone, an accelerometer, or a strain gauge it becomes possible to measure the response

wave as dislocation, velocity, acceleration, stress, distortion, sound***(Atsumi et al., 2007).***

4. Resonance Frequency Analysis (RFA).

Resonance frequency analysis is the most reliable technique to assess stability of dental implants ***(Lachmann et al 2006)***

It is performed using a simple apparatus named "Osstell", which composed of a metal rod which is connected to the implant by screw***(Sennerby and Meredith, 2008)***.

Values range from 0 to 100. High values mean greater stability and low values mean low stability ,ISQ values higher than 65 indicates a successful implantation, ISQ less than 50 indicates increased risk of failure ***(Lachmann et al., 2006).***

5. Percussion test

The percussion test is an uncomplicated method that utilized to assess the osseointegration; it is based on vibrational acoustic science theory and response of dental implant. Type of sound heard after percussion by a metallic tool can detect the clinical evidence of dental osseointegration, where a clearly ringing sound indicates successful dental osseointegration, while a dull sound indicates poor dental osseointegration. The percussion test cannot be used as typical dental osseointegration test because it depend on dentist experience ***(Atsumi et al, 2007)***.

6. Dental Mobility Checker

Evaluation the level of tooth mobility by converting the rigidity of teeth and alveolar bone into acoustic signals**.** Because it might risk the process of osseointegration and it have difficulties of double tapping and the ability to

produce constant excitation so it cannot used immediately following implantation ***(Atsumi et al., 2007; Sandeep et al., 2012***).

7. Periotest

It is used to reveal the damping capacity of peri-implant tissue and the stiffness of the natural tooth or implant by application an electronically and electromagnetically controlled metallic rod connected to a specific hand piece.

The reaction to trapping is registered by a small accelerometer connected to the top of the device. Periotest value (PTV) which is ranged from -8(low mobility) to +50(high mobility) the values from −8 to +9, or 17 degrees indicates success of osseointegration**.** ***Atsumi et al.,(2007) and Mall et al., (2011)*** suggested that the sensitivity of these instruments is inadequate to assess implant mobility because the direction and position of the periotest probe affect the value.

8. Finit element analysis

Finit element analysis is a theoretical analysis, computer simulated, and depend on some properties of material (Young's Modulus, the bone density and Poisson ratio) by using two and three dimensional finite element models **(*Simmons et al 2001*).**

Dalstra et al (2004) stated that stress and strain gained by implants can be studied by finite element modeling.

B. Invasive methods :

1. Cutting torque resistance analysis

It is based on the amount of energy required for an electric motor to cut off a unit volume of bone during implantation (J/mm^3) .Cutting torque resistance analysis was developed to reveal areas of bone with low density as well as to quantify bone hardness during the low-speed insertion of implants **(Wilmes *et al, 2008*).**

2. Push-out/pull-out test

Pull out test is widely used to evaluate stability of dental implant. A relationship between insertion torque and pullout strength has been analyzed and related with screw stability has been studied ***(Huja et al, 2005; Wu et al, 2008).***

Pullout strength increases with increasing in insertion torque up to a point, after that pullout strength decreases and the possibility of failure increases. The decrease in pullout strength happened due to high compression that may causes trauma to the adjacent bone and increases the possibility of failure **(*Cleek et al, 2007).***

3. Insertion Torque Analysis

This method was used to measure the amount of force required to insert implant in bone. Initially minimal torque of insertion, and then increasing rapidly until the cortical layer is fully engaged.The maximum value is gained when the head of the screw makes contact with the cortical plate ***(Motoyoshi et al, 2006)***

4. Histological method

Indirect detection of the stability of implant can be done by examining the bone implant interface. Histomorphometry is a quantitative method which used for measuring the percentage of contact of dental implant to bone tissue from a ground section. Typical parameters are measured includes the bone area within the threads and percentage of bone contact in addition osteocytes and osteoblasts number can be counted ***(Sennerby et al, 1992; Ericsson et al, 2002).***

the drawback of these measurement is requiring of removing of the implant with the surrounding bone, which is clinically unethical, and cannot evaluate the physical properties of bone and tissue, like stiffness around the implant by microscopical picture (***Hsu et al, 2008).***.

5. Reverse torque test

Reverse torque test considered a good diagnostic aid for estimation of osseointegration, based on measuring the higher torque value where bone-implant contact was broken . ***Sullivan et al., (1996*)** stated that reverse torque value more than 20 N.cm considered acceptable for a successful osseointegration. This method is mainly used in experiments because it result in removing of implants **(*Atsumi et al., 2007*).**

Bone surrounds implant may suffer from irreversible plastic distortion due to unnecessary force was applied to an implant that was still undergoing osseointegration **(*Brånemark et al., 1985).***

During removal of implant many factors can influence the torque value such as implant surface structure, implant geometric variations, interface tissue structure, as well as quality and quantity of surrounding bone **(*Sennerby et al., 1992*).**

1.5 Nanotechnology:

Nanotechnology is the science of nano materials it possess the ability to produce benefits in multiple regions such as construction of new materials with superior properties, production technology, information technology and electronics, ecology and energy conservation, medical restorations, transportation, economy, etc. A nanometer is 10^{-9} meter, or one billionth of a meter **(*Lainovic et al., 2012*).**

***Christenson et al., (2007*)** stated that nano materials can be classified according to their form and structure as nanocrystals, nanostructures, nanoparticles, nanocoatings, and nanofibers, Nanotechnology also includes the complicated structures (nanotubes) or one-dimensional concepts (nanodots and nanowires). In present decade, surface modifications of dental implants are extensively depend on nanotechnologies**.** Maximum bone-formation at the bone implant contact is correlated to nanoscale properties **(*Variola et al., 2008*)**

Nano biomaterials compared with conventional materials have greater surface area. Improving of cell attachment and tissue healing when nanoscale material used because it enhance surface wettability to blood and the attachment and distribution of fibrin fibers and matrix proteins on the surface**(*Mendonca et al., 2009)***.

Webster et al in 2003 reported increasing in osteoblast attachment with nanophase materials. Many studies found increase strength of osseointegration for the nano structured surface prepared by physical vapor deposition and increased surface area by about 40 % in comparison to an acid-etched surface. **(*Ogawa et al, 2008*).**

While other studies show that nano HA coated implants placed in rabbits demonstrated enhancing of bone formation **(*Meirelles et al, 2008*)**. And ***Wie et al, (2001)*** report the effect of electrophoretic deposition of nano hydroxyapatite coating on Ti, Ti6Al4V and 316L metal substrate.

Yang and He in 2008 reported the effect of biomimetically and electrochemically deposited nano HA coatings on osseointegration of porous titanium implants.

Waheed in 2013 evaluate the effect of nano ZrO_2 coating mechanically and histologically of screw-shaped Ti-6Al-7Nb dental implants. The results indicated that there was a faster reaction of bone towards the nano ZrO_2 coated Ti-6Al-7Nb alloy implants compared to the uncoated one.

Shukur in 2014 studied the role of nanoscale topographic modification of commercial pure titanium dental implant using a thermal deposition and chemical etching methods for the purpose of improving osseointegration, there was a gradual increase in the removal torque mean values in the studied groups compared with machined group.

Mahmood in 2014 evaluated the effect of pores of nano Hydroxyapatite dual deposits on the bond strength between the living bone and implant. Statistical analysis of the removal torque tests showed highest means for the single nano HA layer at 2 and 4 weeks implantation intervals.

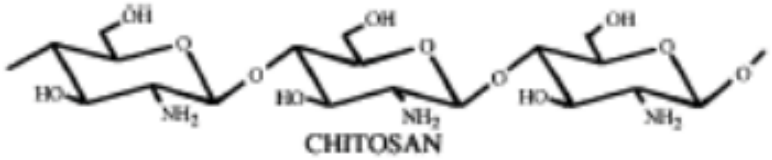
Al-khafaji in 2015 evaluated the use of a composite material which includes (Ceramic nano Al_2O_3 and metallic $AgNO_3$) on the bond strength at bone – implant interface and tissue reaction. The torque mean values at bone-implant interface in coated implants were significantly higher than the uncoated implants at two periods of time (2,4 weeks).

Hussein in 2015 studied the effect of mixture of nano titanium oxide with nano hydroxyapatite coating of screw shaped commercially pure titanium dental implant in comparison to nano hydroxyapatite coating mechanically and histologically at 2 and 6 weeks implantation intervals. The mean removal torque recordings for the mixture of nano titanium oxide and nano hydroxyapatite coated implants was significantly higher than the nano hydroxyapatite coated screws over the two healing periods.

Rafeet in 2016 studied the effect of nano hydroxyapatite and nano zirconium oxide mixture coated on the screw shape commercially pure titanium implant on the strength of bone-implant interface compared to non-coated implants by using torque removal test and histological analysis. The mean of torque removal for implants with coating was higher than implants without coating after (2, 4) healing intervals.

1.6 CHITOSAN :

Chitosan is a natural polysaccharide, chitosan produced by deacetylation of chitin when a level of deacetylation more than 45 % chitosan soluble in organic acids for example acetic or formic acid with stirring, insoluble in water, organic solvents and aqueous bases Brimacombe and Webber in1964 reported that the chitosan composed of repeating units of beta (1-4) 2-amino-2-deoxy-D-glucopyranon(D-glucosamine)(figure1.1) **(*Wilson et al., 2007).***



(Figure 1.1): chitosan structure

The source material for chitosan is the chitin, is the higher abundant organic materials. It is a critical component of the exoskeleton in animals, particularly in shellfish, molluscs and insects. It is additionally the fundamental fibrillar polymer in the cell wall of certain fungi **(*Eugene and Lee, 2003)***

Chitosan is a biocompatible, and non-toxic material, can be categorized as fiber, film, and micro and nanoparticle. **(*Yilmaz, 2004*).**

Chitosan stimulate the differentiation of osteoprogenitor cells so it can accelerate the formation and regeneration of bone**,** the structure of chitosan is same to glycosaminoglycan (GAG) and has many desirable properties for medical uses **(Kumta, et al., 2005).**

Xiong et al. in 2007 study the effect of Preparation of HA/chitosan composite coatings on alkali treated titanium surfaces through sol–gel techniques, resulted in uniform HA/CS coating on Ti surfaces, with good hydrophilicity, which was generally used for bioactivity enhancement

AJAY in 2011 studied the effect of hydroxyapatite and hydroxyapatite-chitosan composite coatings on stainless steel by electrophoretic deposition method, resulted in homogenous coating.

1.6.1 *APPLICATIONS OF CHITOSAN:*

Chitosan is considered as effective decision for biomedical applications, which incorporate wound healing, vaccine delivery, blood coagulant, digestive sutures, in artificial kidney membrane, hypercholesterolemic agents, and hemostatic agent and tissue regeneration.

In dentistry chitosan is used, to inhibit the formation of plaque and tooth decay. Chitosan can be utilized for treating periodontal diseases like gingivitis and periodontitis because it has ability to recover the connective tissue that covers the teeth close the gums **(*Wilson et al., 2007).***

A. ***Skin:***

Chitosan utilized as artificial skin substitute without adverse impact taking after implantation in tissue, with normal course of healing with formation of normal granulation tissue**.** Chitosan has numerous advantages for wound healing include hemostasis, rapid tissue regeneration and activating fibroblast for production of collagen ***(Mi et al., 2001).***

B. ***Bone substitutes:***

Chitosan powder used to encourage healing of periodontal pockets, palatal wounds as well as extraction places (***Kim et al., 2008***).

Martino et al., (2005) and in 2008 Kim et al., reported that chitosan has been used in bone tissue engineering, and has ability to improve growth and mineral rich matrix deposition by osteoblasts in culture.

C. ***Anti-bacterial:***

The antibacterial activity of chitosan against S. aureus demonstrated a high growth inhibition about 80% ,and have antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Methicilin-Resistant Staphylococcus Aureus (MRSA)*, and *S. aureus***(*Hu et al., 2003).**.

1.7 COLLAGEN:

Collagen is a Greek word where “kola” means gum and “gen” means producing. Collagen is a basic structural protein exists in the extracellular matrix and connective tissue of animals. About 25% of the protein content of the whole body particularly in mammals is the collagen, which present in the bones, blood vessels, cartilage, and dentin of teeth. Fibroblast of connective tissue is responsible for production of collagen (***Silvipriya et al., 2015)***

Collagen has excellent biocompatibility, low antigenicity, and low inflammatory and cytotoxic responses; therefore the use of collagen in biomedical application has been rapidly developing and widely extended to bioengineering areas **(*Kadler et al., 2007).***

There many types of collagen (About 19 types). All types of collagen consist from three polypeptide chain arranged in the form of a triple helix with two identical chains (α1) and the third which differs to some extent in its chemical composition (α2). Each chain contains about 1000 amino acids, twisted around each other in a common right-handed helical structure which is 300 nm long. Collagen promotes protection to skin by preventing the absorption of toxins and pathogens ***(Fratzl, 2008***).

It influences biological functions of a cell (cell survival, proliferation and differentiation); it assists in healing of damaged tissues, bones or blood vessels and preserving structural integrity (***Silvipriya et al., 2015).***

Jamil in 2011 Compare of the influence of the implant coated by biological material(collagen),and implant coated by both bio-inert ceramic(zirconia) and(collagen), on osseointegration at (3days,1, 2and6weeks healing intervals) by immunohistochemical , and radiographical studies with mechanical test. Resulted in that coating of implant with collagen and coating with both PSZ and collagen showed an increment in osseointegration in short interval period.

Masayoshi et al. in 2013 studied bone formation around three groups were prepared: un coated titanium rod specimens, hydroxyapatite (HA) coating, and hydroxyapatite/collagen (HA/Col) nanocomposite coating, each specimen was placed under the periosteum of a male rat calvarium. Four weeks after surgery, the samples were evaluated via histomorphometrical analyses and bonding strength tests. All the uncoated specimens and more than half of the HA specimens were encapsulated with fibrous tissue, whereas all the HA/Col specimens were almost completely surrounded by new bone tissue without encapsulation.

Dolly et al. in 2014 studied the role of Collagen type-I coating on Magnesium– Zirconia (Mg–Zr) alloys, containing different quantities of Strontium (Sr), in enhancing the in vitro bioactivity and in vivo bone-forming and mineralisation properties of the implants, resulted in Sr content and Col-I coating of Mg–Zr–Sr alloys significantly improved their bone inducing activity in vitro and in vivo.

Sang et al. in 2014 were compared peri-implant bone formation among uncoated (UC), hydroxyapatite (HA), collagen plus HA (CH), and collagen, HA, plus bone morphogenetic protein- 2 (BMP-2) implant groups. In this study, the (CH) group displayed significantly greater new bone formation than the other groups. There was no significant difference among the other groups.

1.7.1 *Sources of collagen* :

Collagen has been taken out from animal tissues (collagen not present in plants), for example; extracted from skin and bone of cow (bovine), which considered major industrial sources of collagen. Collagen can extracted from the skin and bones of pigs (Porcine**)**, rabbit femur, rat, from human tissue like placenta, marine invertebrates and vertebrates such as fishes, star fish, jellyfish, sponges, sea urchin, octopus, cuttlefish, and prawn (***Silvipriya et al., 2015).***

1.7.2 APPLICATIONS OF COLLAGEN:

1. ***Pharmaceutical industries***

Collagen is extensively utilized in pharmaceutical industries because low antigenicity, cell attachment capability, biodegradability and biocompatibility (***Silvipriya et al., 2015)***

2. ***Dental field***

Clinical researches have reported that collagen exhibit the ability of improving wound healing after dental therapy by clot formation and stabilization, so it act as a natural hemostatic agent**.**

Collagen can be used for endodontic repair, Collagen have been widely used in periodontal and implant treatment as barriers that inhibit the migration of epithelial cells and stimulate wound regeneration by cells with regenerative potential.(***Ruby et al., 2013).***

3. ***Medical field***

Collagen plays an important role in medicine presently. It aids in cartilage and bone regeneration. In vascular and cardiac reconstruction, collagen in the form of tissue engineered blood vessels is implanted successfully in the patients. Collagen utilized as dressing in the form of sponge for wounds or in the form of collagen films and powders for burns, in urogenital disorders and corneal defects **(*Silvipriya et al., 2015).***

Chapter Two

Material And Method

Chapter Two

2.1 In vitro experiments:

2.1.1 Materials

1. Commercially pure titanium rod (CpTi) rod grade (II) (29mm diameter, 31cm length) (Orotig S rl EU Company, Italy).
2. Hydroxyapatite powder (Particles size 98.5%, <40nm), sky spring nanomaterial, USA.
3. Chitosan powder (degree of deacetylation 85%) (Xian Lyphar Biotechnology Co. china).
4. Collagen powder (C5608, SIGMA ALDRICH, USA).
5. Ethanol absolute 99.8%. (SIGMA ALDRICH, Germany).
6. Distilled water.
7. Phosphorous pentoxide P_2O_5 (Emphos PS-21A, Witco).
8. Silicon carbide paper.
9. Nitric acid (HNO_3) and Hydrofluoric acid (HF) (Biosolve ,UK)..
10. Argon gas(Iraq).
11. Acetic acid 2% (Scharlau S.L., Spain).

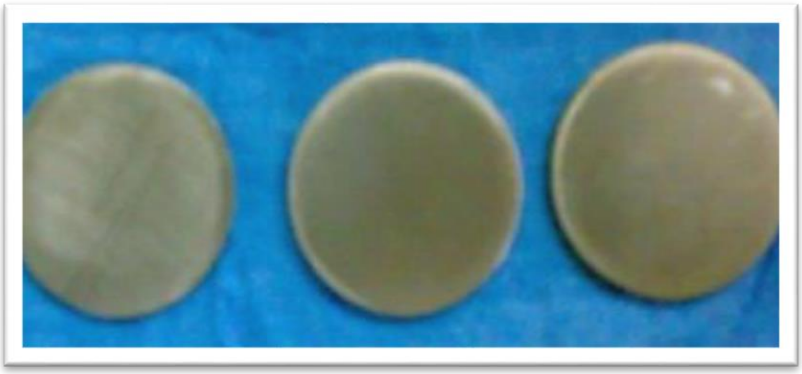
2.1.2 Equipments

1. Analytical balance ±0.0001 accuracy (Sartorius, India).
2. Hot plate stirrer (Daihan Lab Tech/Model: LSM-1003, Korea).
3. Tube furnace. Carbolite type MTF 12/38 A. Bamford England. Serial No. 3/88/432.Maximum temperature 1200 $^{\circ}$C.
4. Optical microscope (Nikon Eclipse ME 600L/441002, Japan) provided with digital camera type DXM 1200 F. Nikon ACT Version 2.62, 2000 software.

5. Micro process coating thickness gauge (Erichsen GMBH & CO KG, D-5870 hemer sundwig, W-Germany).
6. Ultrasonic cleaning bath (Sonomatic/170-2-T80, Germany).
7. X-ray diffractometer (Shimadzu, XRD-6000, Japan).
8. FTIR Spectrometer (Biotech, FTIR-600, UK)
9. Scanning probe microscope (AA300 Angstrom Advanced Inc ,USA)
10. Stop watch.
11. Glass containers.
12. Lathe machine.
13. Rotative grinding and polishing machine.

2.1.3 Sample preparation

Commercially pure Titanium (grade 2) was cut into small circular discs (29 mm diameter and 2 mm thickness) with a lathe machine then used as the substrate for coating, as shown in (**figure 2.1).**



(Figure 2.1): Commercially pure Titanium (cp Ti) discs

These disks were grinded by silicon carbide paper starting from 500 to 1000 grit, a rotative polisher at 250 rotations per minute (rpm) for 2 minutes for each step of grinding till uniform smooth surface was obtained.

The disks were cleaned by using a solution composed of 30% of HNO_3 and 10 % of HF dissolved in (60%) distilled water as shown in (**figure 2.2)**, then washed with distilled water, ultrasonic cleaning with ethanol was performed for

15 mins. to remove the contamination and debris from the polished discs as shown in**(figure 2.3)** ,then the specimens were dried at 100 ºC for another15 minutes finally the discs were washed with distilled water ***(Jani, 2014).***

(Figure 2.2): cleaning of discs

(Figure2.3): ultrasonic cleaner

2.1.4 Pilot study

I. Suspension preparation

A. Preparation of Nano-HA coating solution

The coating suspension of nano hydroxyapatite consists from dissolution of 0.01g of P_2O_5 in 50 ml of absolute alcohol (ethanol) and heating at (45ºC) on a hot plate stirrer for half an hour ***(Jamil, 2011).***

Then (7g) of nano-hydroxyapatite powder was then added to the solution, the temperature was to be maintained at approximately 45°C then the mixture was left over a stirrer to gain homogenous solution as shown in **figure (2.4)** ***(Jamil, 2011; Salman, 2011 and Hussein,2015).***

(Figure2.4): Nano HA solution

B. Preparation of Nano HA/CHITOSAN composite coating solution:

1-(4g)of nano-HA and (0.5g) chitosan were added to beaker containing 48 ml water and 5ml ethanol the mixture was stirred to form homogenous mixture(***Zhenyu, et al., 2015)***, the chitosan did not dissolve and it participated in the bottom of beaker**(Figure2.5).**

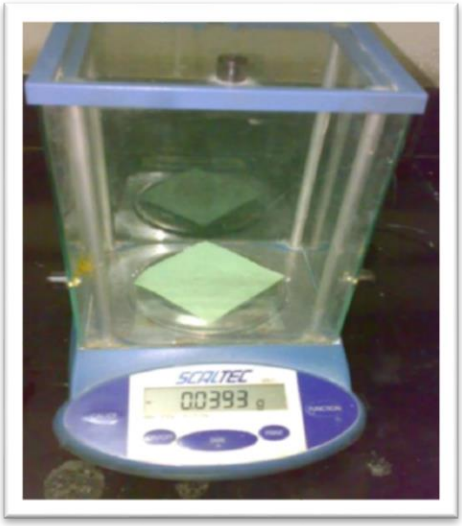
(Figure2.5): chitosan not dissolved in water mixed with ethanol

2-The chitosan (0.5g) dissolved in 50 ml of 2% acetic acid (***Lu et al., 2006***), which it was mixed with the nano-HA solution (4g) dissolved in 50 ml ethanol **(figure 2.6).**

(Figure2.6): chitosan solution mixed with nano HA solution

C. Preparation of Nano HA, Chitosan and Collagen composite coating suspension:

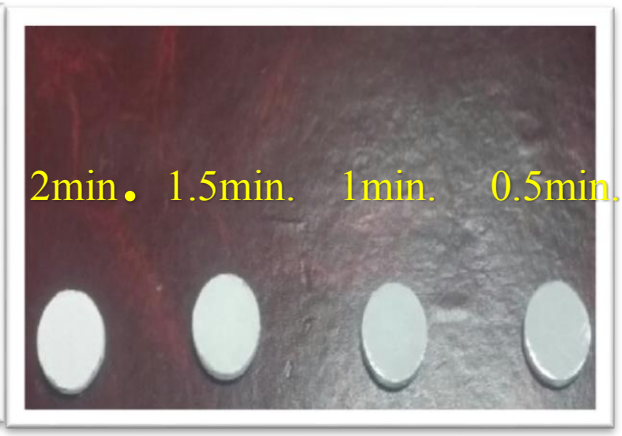
(1mg/ml) of collagen was added to chitosan solution and then mixed with nano-HA solution, and the solution was left over a stirrer for half hour until homogenous mixture obtained. The material weighting was done by using analytical balance **(figure2.7).**



(Figure 2.7): Analytical balance

II. Disc coating

The coating was done by immersing the disc that hold with tweezer in the mixture suspension for (30 seconds) and withdrawn with a well-defined withdrawal speed, and dried for one minute at room temperature and then returned it to the coating suspension best result of coating was obtained at 2min., as shown in (**figure 2.8), (figure 2.9) (*Jamil 2011).***

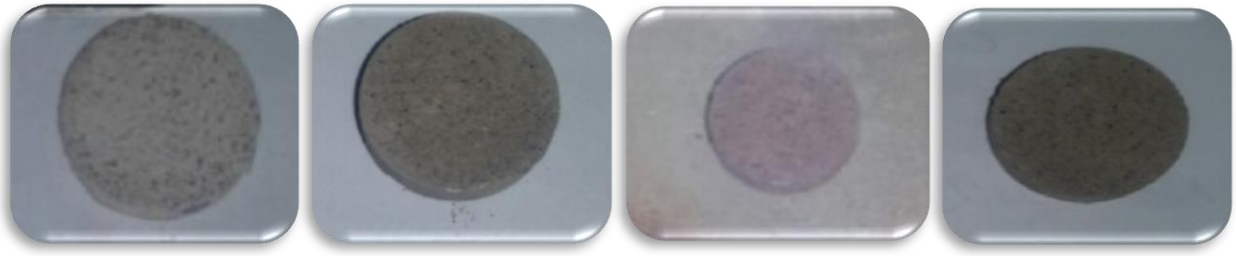


(Figure 2.8): disc coating *(Figure2.9): disc coating at different time*

III. Heat treatment

Heat treatment of the nano-HA coated specimens (sintering) was carried out for densification using Carbolated furnace (tube furnace) under the presence of an inert gas (argon)**(figure2.10**), to prevent oxidation of the specimen. Best results were obtained at 400°C because at a temperature less than 400°C the coating layer was highly porous, less dense while at higher than400°C, the coating was with noticeable cracks **(Sonawane et al, 2002)**. On the other hand sintering of nano-HA/chitosan composite coated specimens was tried at (400-300-200-250-150-100-60) °C, to select the appropriate heat temperature. Best results were obtained at 100°C because at low temperature the coating layer was highly porous less dense, while sintering of specimen more than 100°C, resulting in burning of coated material ***(Figure 2.11).***

(Figure2.10): Carbolite furnace



(A) 400ºC *(B) 300 ºC* *(C) 250 ºC* *(D) 200ºC*

(E) 150ºC *(F)100 ºC* *(G) 60 ºC.*

(Figure 2.11): Heat treatment of nanoHA-chitosan composite coated discs

2.1.5 Tests performed

2.1.5.1 Thickness Measurement

All coated samples thickness were measured by using (Erichsen Mini test 3000) microprocessor coating thickness gauge, (**figure 2.12).** This test is non-destructive method for coating thickness measurement. Measurement taken by choosing three points on the coated layer first point on the border of disc and other points was chosen on the same line toward center of disc then we calculate the average of these measurement which consider the thickness of coated layer.

(Figure 2.12): Digital gauge for coating thickness

2.1.5.2 Microscopical Examination

Examination of the surface feature of coating was done using optical microscope (12 samples) (Nikon Type 120, Japan optical microscope) resulted in a series of micrographs to show microstructure of coated layer **(Figure 2.13).** The microscope was provided with digital camera type DXM 1200 F. The micrographs were analyzed through Nikon ACT- version 2.62, 2000 software.

(***Figure 2.13): Optical Microscope with digital camera***

2.1.5.3 X-Ray Phase Analysis

Phase analysis was employed on samples before and after coating with different materials. Shimadzu Lab XRD – 6000 Powder X ray diffractometer using Cu Kα radiation was used to examine the phase analysis **(Figure 2.14)**. The 2Ɵ angles were swept from 0- 70º in step of one degree. The peak indexing was carried out based on the JCPDS (joint committee on powder diffraction standards).

(Figure 2.14): X-ray Diffractometer device

2.1.5.4 Surface Analysis (Scanning Electron Microscope SEM)

All experimental groups were imaged using scanning electron microscope, this device was used for determining the surface morphology and topographical characteristics of coated specimen. In SEM technique, an electron beam is scanned over the sample surface.

2.1.5.5 FTIR analysis

It is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high spectral resolution data over a wide spectral range (***Griffiths etal 2007).***

The infrared (IR) spectra were performed on Biotech FTIR-600 Main Spectrometer, to determine the interaction between nano HA, chitosan and collagen (***Figure 2.15).***

(Figure 2.15): FTIR spectrometer

2.1.6 Implant preparation

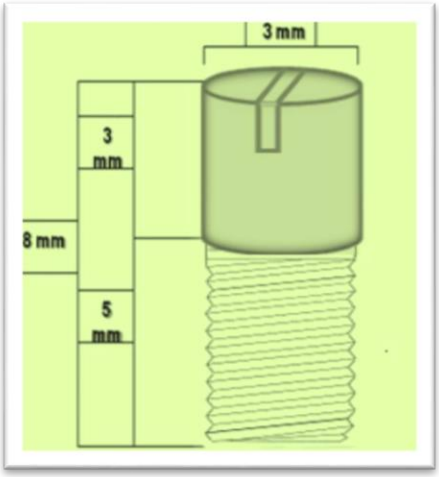
1. Materials and Equipment

The same materials used in the vitro study in addition to the Gamma cells for sterilization and CpTi rods machined into screw shaped implants.

2. Methods

(fifty four) screw shaped implants, 3.0 mm in diameter and 8mm in length (threaded part is 5mm and smooth part is 3mm) and pitch height is 1mm, were machined from CpTi rods using Lathe machine .The head of the implant had a slit to fit the screwdriver during insertion and removal by torque meter during mechanical testing(**Figure 2.16, 2.17**) .

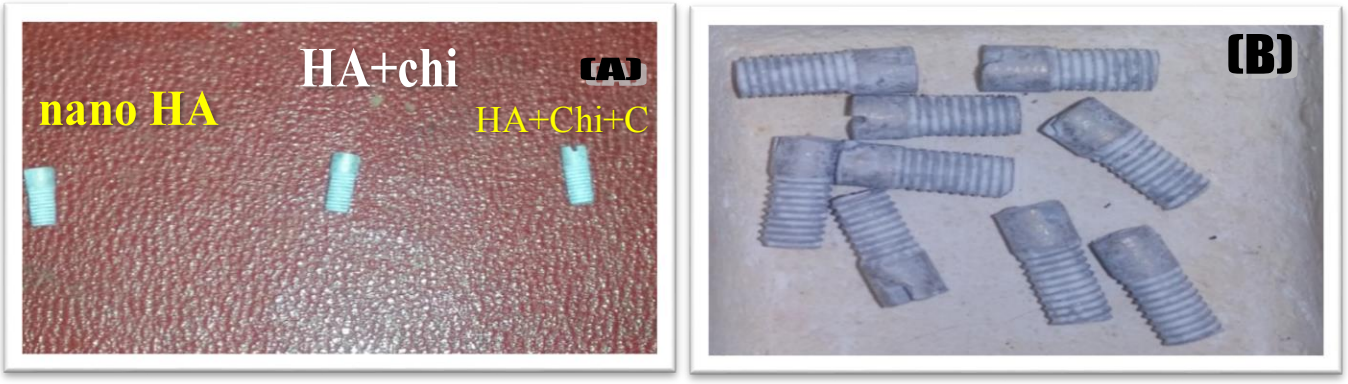
Screw was cleaned by using a solution consisted of 30% of HNO_3 and 10 %of HF in distilled water this solution to remove the oxide layer from the surface and the contamination then screws were washed by distilled water. After that screws were placed in ethanol for 15 minute in an ultrasonic cleaner bath then washed by water, and dried in an oven at 100° C for 15 minutes ***(Jani, 2014).***



(Figure 2.16): implant design (Jani 2014)

(Figure 2.17): machined Screws

Then the screws were divided into three groups each group consisted of 18 screws. The first group of screws was coated with HA , while the second coated with HA -chitosan composite and third group was coated with HA –chitosan-collagen composite, following the same procedure of dip coating that was performed on disc specimens **(pilot study)(figure 2.18)**

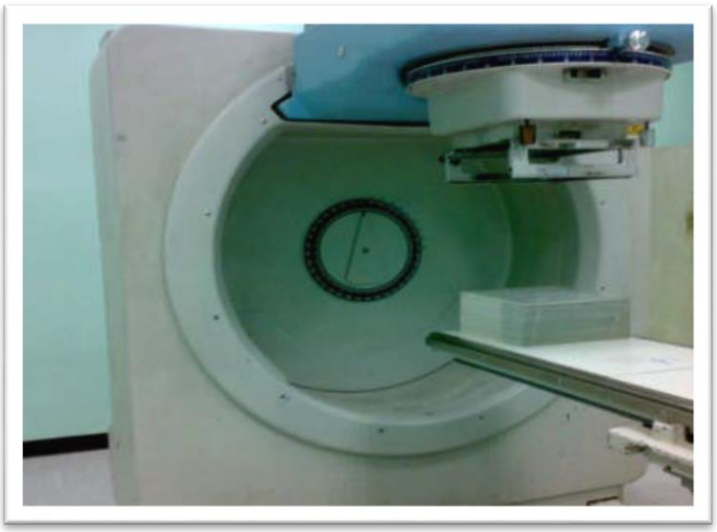


(Figure 2.18 A,B): coated screws

3. ***Sterilization:***

Each screw was kept in single airtight sheets before sterilization (**figure 2.19)**. Gamma radiation dose of 2 mega rad using gamma cell 220 with a Co^{60} source used for Ti screws sterilization. This dose is required for medical and surgical equipment sterilization. This procedure was done in the radiation department of Al *Amal Hospital*, according to the atomic energy of Canada limited (AECL, 1984). The radiation used having energy of about 1.25 MeV (Million Electron Volts) with a dose rate of 90.4 rad/min and 65 cm distance between screws and a source of radiation, the exposure time was for 60 minutes **(figure 2.20).**

(Figure 2.19): coated sterile screws



(Figure2.20): Gamma Radiation Device

2.1.7 Sample grouping:

The coated screws were subdivided into 2 groups according to the test performed, (**Figure 2.21)**.

1. ***Mechanical (torque measurements):*** **(36** screws) the screws were divided into:

a. Control group (**12** screws): coated with nano HA for each healing period (2 and 6weeks).

b. Experimental group (**24** screws):

I- 12 screws for HA/chitosan group

II-12 screws for HA/chitosan and collagen group

This group included 6 screws for each healing period (2 and 6weeks).

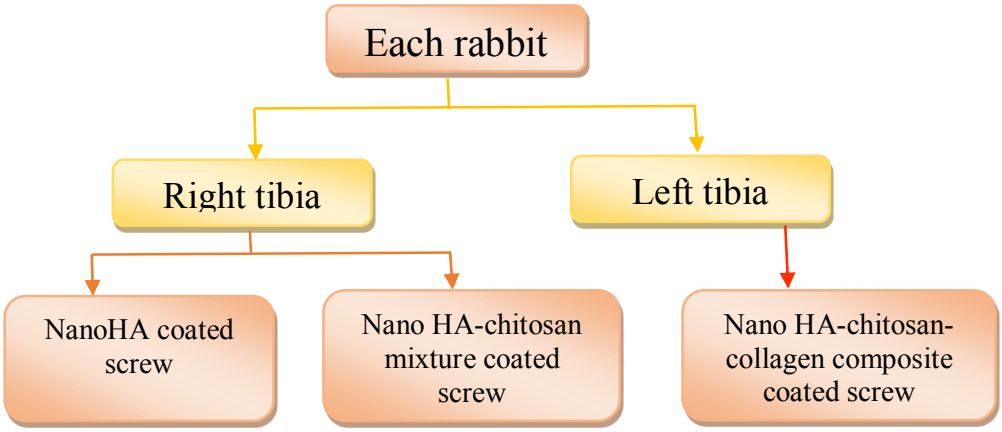
2. ***Histological test*: (18** screws), in this test the screws were divided into:

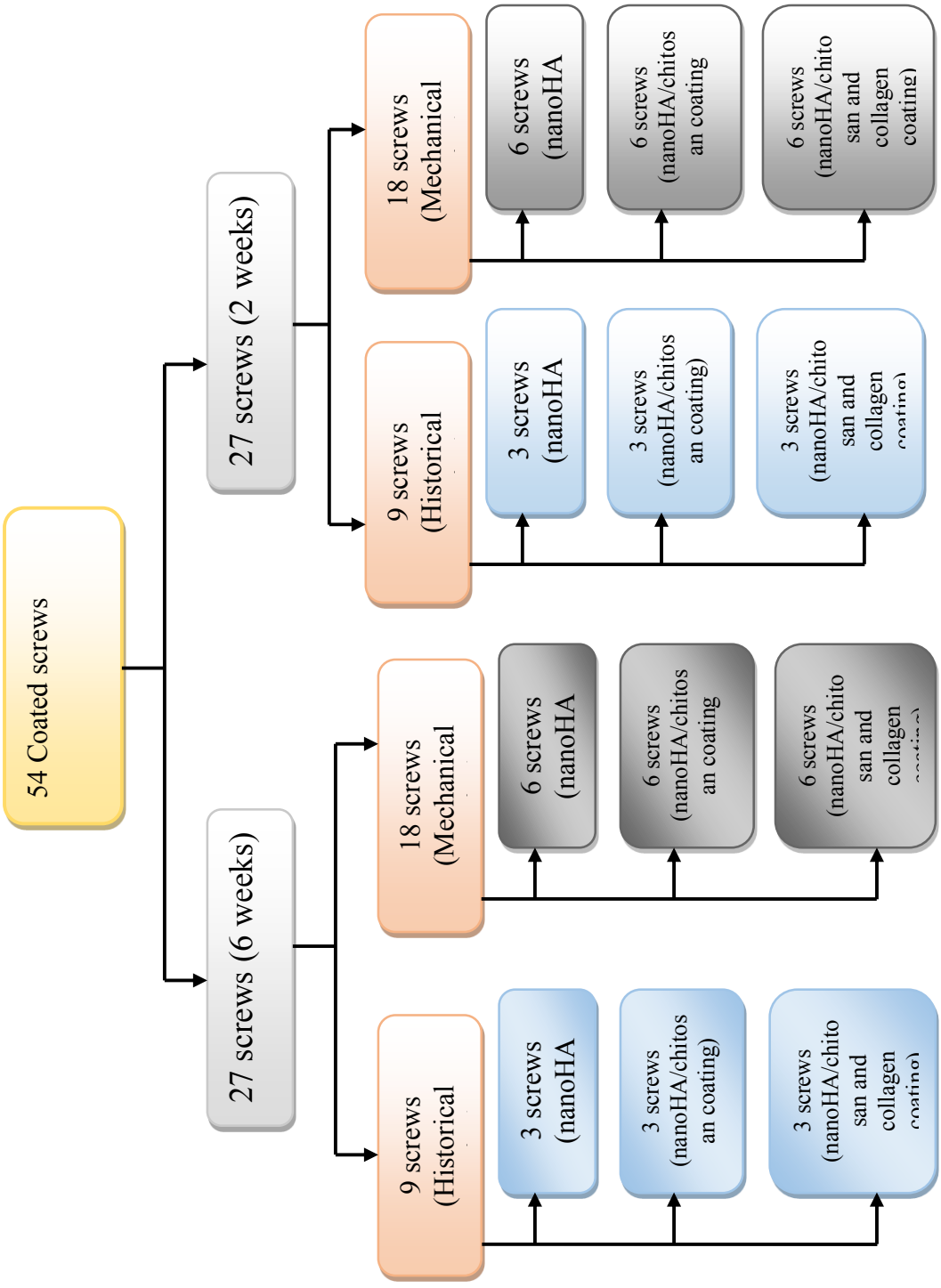
a. Control group (**6** screws): including 3 screws for each healing period (2 and 6 weeks), coated with nano HA.

b. Experimental group (**12** screws):

I-**6** screws for HA/chitosan group: includes 3 screws for each healing period (2 and 6 weeks)

II- 6 screws for HA/chitosan and collagen group: includes 3 screws for each healing period (2 and 6 weeks)





(Figure2.21): Experimental design of the in vivo experiments

2.2 In vivo experiments

2.2.1 Animal Preparation and description

Eighteen healthy adult New Zeeland male rabbits, weighing 2 -2.5 kg, about11 months age were used. Animals were kept in standard separate cages and had free access to tap water, and were fed with standard pellets **(Figure 2.22)**.

To ensure parasite free animals Intramuscular, Ivermectin injection was given also an antibiotic cover with oxytetracycline intramuscular injection was given for 3 days to exclude any infection. The rabbits were then left for two weeks in the same environment before surgical operation**.** All this work done at the College of Veterinary Medicine/ University of Baghdad for about 2months (from *April* to *June* 2016).

(Figure 2.22): Experimental animal

2.2.2 Surgical procedure and implantation

2.2.2.1 Materials and instruments

1. Alcohol 70% (Iraq).
2. Anesthetic solution (Ketamine 100 mg (10%) Holland) and (Xylazine 20mg 2% Belgium).
3. Catgut suture. (3/0 DyNEK sutures, Australia).
4. Cotton and gauze.
5. Disposable non-woven bed covers (China).
6. Disposable syringes.
7. Drills (Densply,Maillefer,SWISS)
8. Iodine 10% (Lebanon).
9. Ivermectin 10mg (Syria).
10. Long-acting oxytetracycline(Kepro Oxetet 20%, Holland).
11. Needle holder (Germany).
12. Normal saline solution (0.9%Haidylena /Egypt).
13. Occlusal X-ray film (Kodak).
14. Scalpel with blades.
15. Scissors (China).
16. Screwdriver(China)
17. Silk suture (3/0 HUAIAN ANGEL medical instruments co.,LTD.CHINA).
18. Surgical drapes (Iraq).
19. Disposable gloves (Malaysia) and Surgical gloves and masks (China).
20. Shaving spray (Gillette, UK.)
21. Topical aerosol (spray) oxytetracycline (OTC, Iran).
22. Tissue forceps**.(figure 2.23)**

(Figure 2.23): materials used in surgical procedure

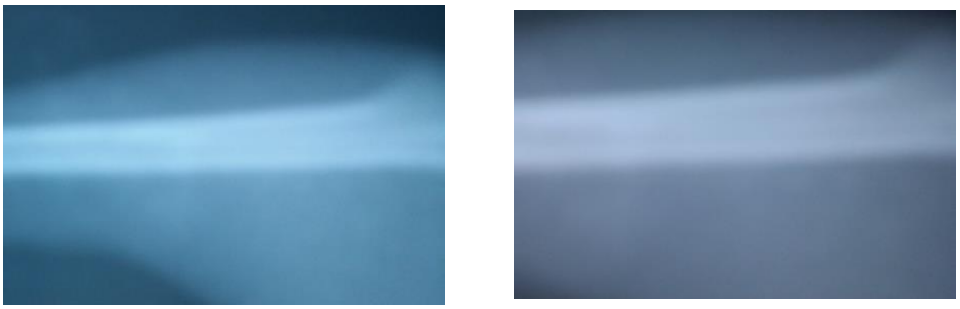
2.2.2.2 Equipments

1. Autoclave.
2. Balance (1-50 g).
3. Engine.
4. Conventional X-ray machine (TOSHIBA, Japan).
5. Torque meter (0 -70 inch. ounces) (Dentium, Korea).
6. Implant ratchet TBR **(Figure 2.24).**

(Figure 2.24): torque meter and implant ratchet

2.2.2.3 X-ray Examination

The operation site was twicely radiographed using conventional X-ray machine, for a 2 sec., the radiation dose was 0.150 mSv at a distance 8 inch away from the object. The first exposure was taken prior to the surgery to ensure the presence of a sufficient bone for implants (**figure 2.25)**.while the second radiograph was done after six weeks.



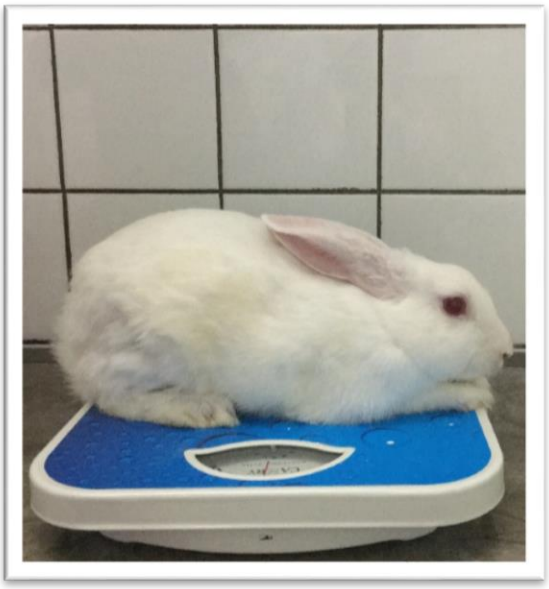
(A) Right tibia (B) left tibia

(Figure2.25): X-ray prior surgery

2.2.2.4 Methods

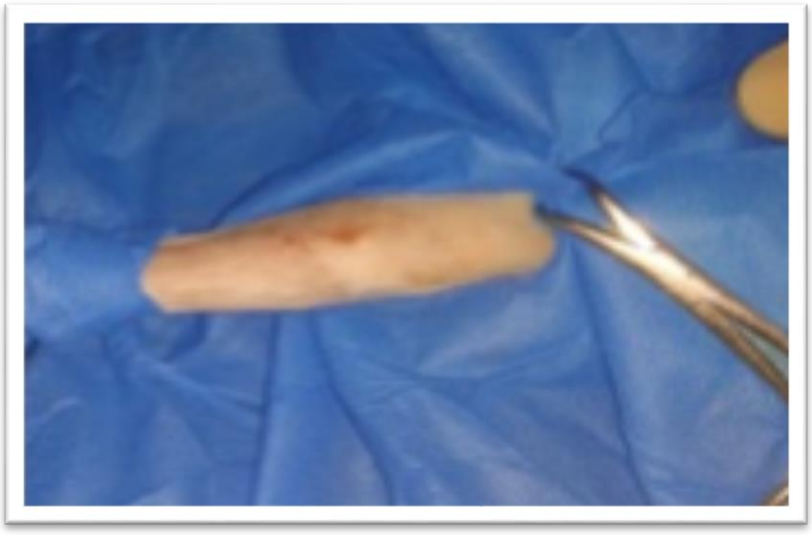
All instruments and towels were autoclaved at 121 C˚ and for 30 minutes. Each animal was weighed before operation to determine the required dose of anesthesia and antibiotic (**figure 2.26**). Anesthesia was given by intramuscular injection of ketamine hydrochloride10% (1ml/kg body weight) and Xylazine 20% (1ml/kg body weight) Diazepam 5 mg (1ml/kg body weight), the dose of antibiotic and anesthesia was calculated according to the following formula:

$$dose = \text{animals weight} \times \text{dose rate} \div \text{constration of drug}$$



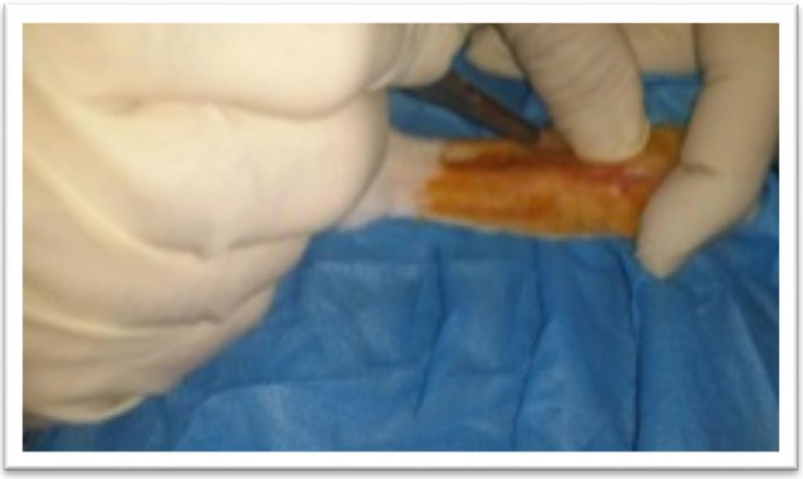
(Figure 2.26): Weighing of the experimental animal

The operation was performed under aseptic conditions**.** The medial sides of both tibia were shaved by using a shaving spray and then the skin was cleaned with ethanol **(figure 2.27),** after that the surgical towels were placed around the operation site.

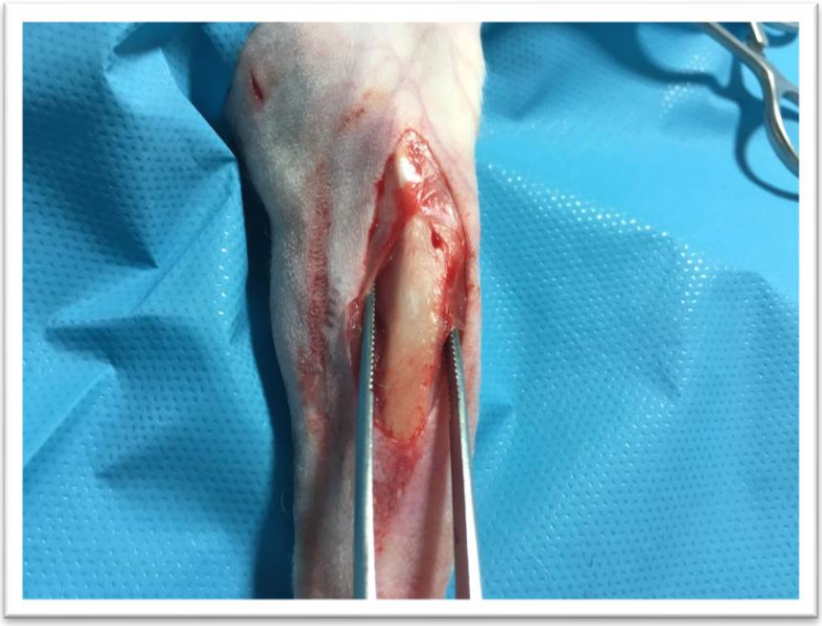


(Figure 2.27) shaved skin

Later on, the incision was made to expose the medial side of the tibia, then reflection of skin and fascia flap was done, **(figure 2.28), (figure 2.29).**

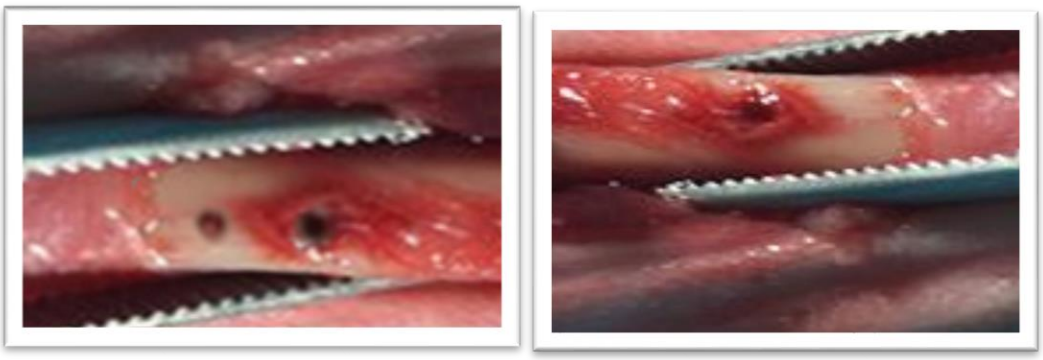


(Figure 2.28): Incision to expose bone of tibia

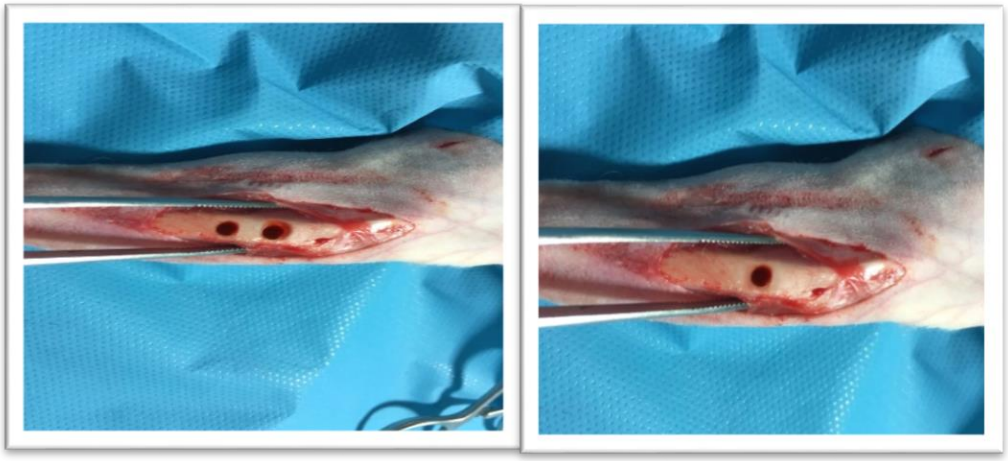


(Figure2.29) reflection of skin and fascia flap

Bone penetration was done with a round drill of 2.0mm in diameter to make two holes with 10 mm distance between them, with intermittent pressure and continuous cooling and normal saline at a rotary speed of 1500 rpm and reduction ratio 16:1. Gradual enlargement of these holes was made with other round drills to 2.8mm. Finally, washing of the operation site with saline to remove debris **(figure 2.30, 2.31).**

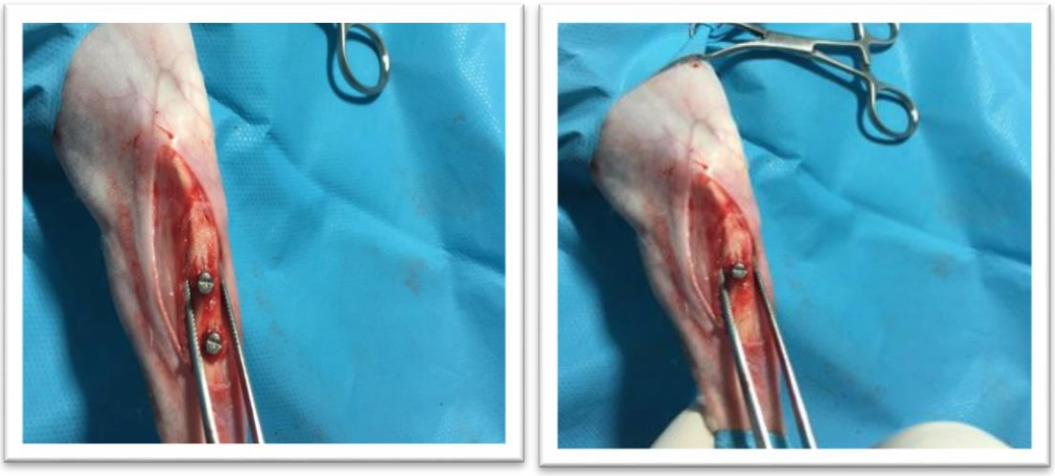


(Figure 2.30): initial penetration of bone



(Figure 2.31): enlargement of holes

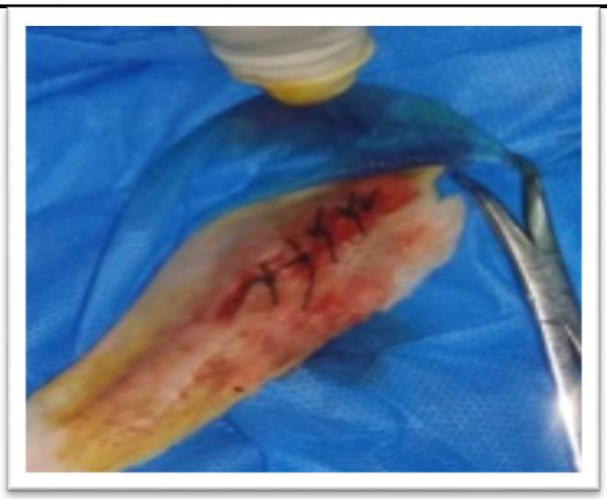
Nano HA coated screw was removed from air tight plastic sheet and placed in the first hole in right tibia (proximal one) by employing screw driver of torque meter that set inside the screw slit until 5mm of the screw was completely inserted into the bone (10N.cm) and verified for stability. The second screw (coated with mixture nano HA and chitosan) was placed to second hole (distal one) (10N.cm). after which both implants were rinsed with saline, the mixture of nano HA-chitosan and collagen coated screw was inserted in hole in left tibia(10N.cm)then the field was rinsed with saline (**figure 2.32) and (figure 2.33).**



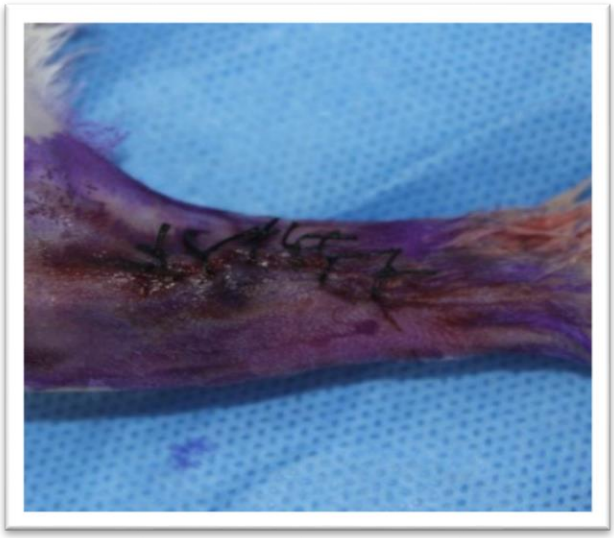
(*Figure 2.32): screws in position*

(Figure2.33): using of torque meter during positioning of implants

An absorbable catgut suture 3/0 was used for muscles suturing then skin suturing with silk suture 3/0**, (figure2.34).** Local antibiotic (oxytetracycline spray) was applied to the surgical site (**figure2.35**). Systemic antibiotic (oxytetracycline 20%, 0.5ml/kg body weight.) was given then the experimental rabbits where transferred to their cage.



(Figure2.34): muscle suturing

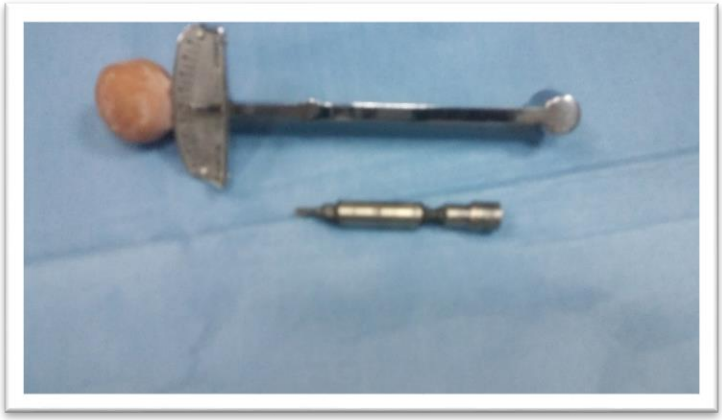


(***Figure2.35): local antibiotic***

2.2.3 Mechanical Testing (Torque test)

2.2.3.1 Materials and Equipments

With a sterile surgical tools and anesthesia similar to that used during the implantation process. Torque meter was used to perform this procedure (Sturtevant Richmont torque product, Model F 80-1-0. USA) **(Figure2.36)**

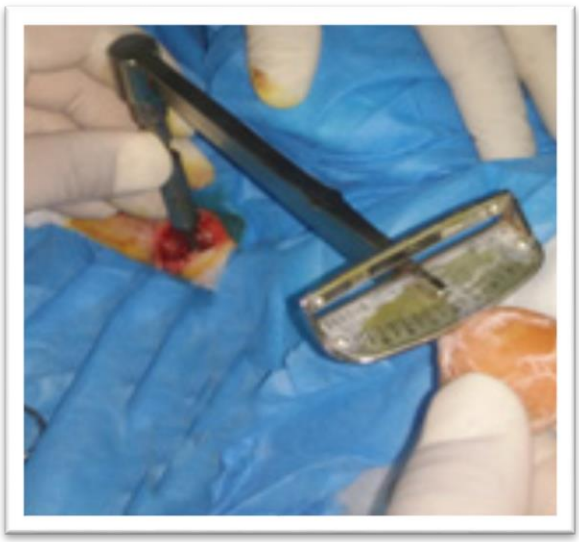


(Figure2.36): Torque meter

2.2.3.2 Method

From each healing interval 6 rabbits were used for torque removal measurement. The measurement was applied after injecting the animal with anesthesia in the same manner performed for the surgical implantation.

Deep incision was made over the skin covering the tibia then fascia and muscles were separated to expose the screws. After that the soft tissues were excised from the rabbit tibia. Checking the screw stability by hand instrument ends. The bone was supported firmly during application of this test to prevent movement. The test was performed by insertion the torque screw driver inside the slit of the screw to record the highest torque needed to remove the screw from its bed as shown in (**Figure 2.37).**



(Figure2.37): removal of screw with torque meter

2.2.4 Histological testing

2.2.4.1 Materials and equipments

- Using sterile surgical tools and anesthesia similar to that used during the implantation process.
- Disc and mandrel.
- Optical microscope (Olympus /542037, Japan).
- Prosthetic engine (Marathon Saeyang Microtech, Korea), (**figure 2.38).**
- Histological and chemical materials:

A. Canada balsam (Batch NO. 10862501, European Union).

B. Distilled water.

C. Ethanol alcohol 96%.

D. Formaldehyde 37%.

E. Formic acid 10% (Batch NO. 28380, England).

F. Haematoxylin and eosin (H&E) (Dako, U.S.A).

G. Microscopic glass slides and covers (China).

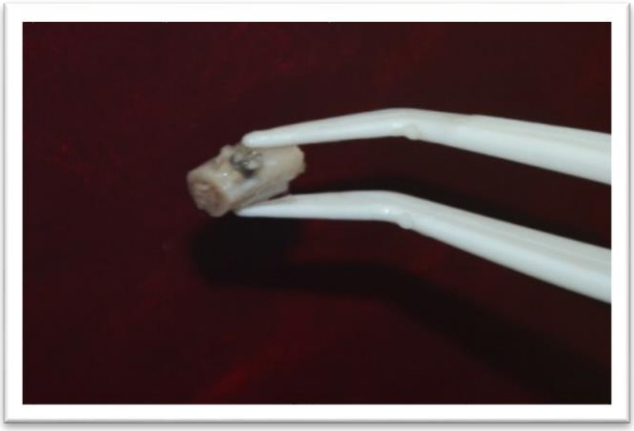
H. Paraffin wax (Hinweis, U.K.D).

(Figure2.38): prosthetic engine

2.2.4.2 Method

From both 2nd and 6th weeks healing periods 3 rabbit anesthetized by overdose anesthetic solution and then scarified for histological examination by optical microscope. A disc with slow speed handpiece rotation and cooling saline was used for bone tissue cutting around the screws (**Figure2.39**). Cutting was performed 5 mm away from the screws in preparation of a bone implant block for histological study (**Figure2.40**).

(Figure2.39): Cutting of bone to prepare bone implant block



(Figure2.40): bone implant block

Bone-implant blocks were immediately kept in 10 % formalin solution for 3 days for fixation (**Figure2.41).** When fixation was completed a series of laboratory procedures were carried out.

(Figure2.41): bone implant block stored in 10% freshly prepared formalin

Then the implant-block was immersed in 10% formic acid for bone tissue decalcification which takes 2-3 weeks, this was usually checked by penetration of a narrow needle to the deepest part of the bone-implant block **(Bhaskar, 1991)**.

After that the bone block divided longitudinally parallel to implant with a small scalpel into 2 equal parts with the implant remaining in one of them, then the implant was removed from its socket slowly.

Dehydration of the bone tissue was done by immersing it in alcohol with serial concentrations (70%, 80%, 90% and absolute alcohol for 60 minutes in each concentration)

The bone tissues then were passed through two changes of xylene for 15-20 min. Each specimen was putted in the center of melted paraffin dish **(Figure 2.42)** and the dish was placed inside a constant-temperature oven regulated to 60 ºC for half an hour. Paraffin were used for immersion, so that paraffin replace all of the xylene in the tissue ***(Linder, 1985)***.

The moulded specimens were adjusted to the microtome where serial sectioning about 5 µm of thickness for each section was performed and placed on a slide.

A Total of 10 sections were made for each block of coating material and for each time interval. Staining the tissue was done by placing the slide in a container having haematoxylin and eosin stain for 10 minutes.

By light microscope with SAMSUNG, GT-N7100 camera, Photographs of each section were taken.

(Figure2.42): paraffin block

2.2.5 Statistical analysis

For this study a suitable statistical methods were applied for analyzing and assessment of the results, including:

2.2.5.1 Descriptive statistics

1. Statistical tables.
2. Summary statistic of the readings distribution (mean, SD, SE, minimum & maximum).
3. Bar –chart for graphical presentation.

2.2.5.2 Inferential statistics

These were used to accept or reject the statistical hypotheses. Testing the quality of means by T test, ANOVA and LSD was used to compare the significance difference between means in this study.

Note: The comparison of significant (P-value) in any test were:

S= Significant difference ($P \leq 0.05$).

HS= Highly Significant difference ($P \leq 0.01$).

NS= Non Significant difference ($P > 0.05$).

Computer programs

All the statistical analysis was completed by using SPSS program (version-23) and Excel software.

Chapter Three

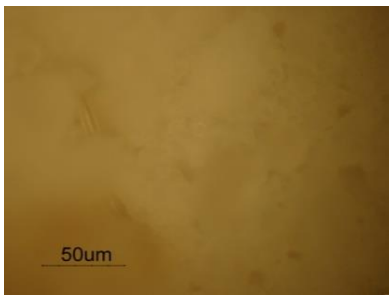
Results

Chapter Three

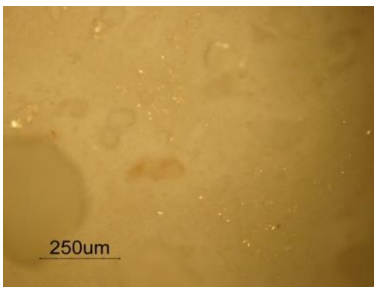
3.1 In vitro experiments:

3.1.1 Optical microscopical observation

A series of micrographs illustrate the microstructure of Nano HA(Figure 3.1), Nano HA-chitosan composite (Figure 3.2) and mixture of Nano HA –chitosan- collagen composite coated cpTi surfaces (Figure3.3) for different times under different magnification powers ($50x$, $100x$).A homogeneous thickness layer over the substrate of cpTi during coating for 2 min with completely cover of substrate.

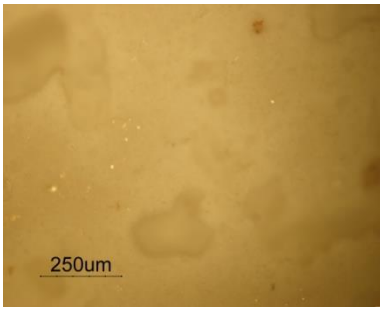


30sec., $50x$

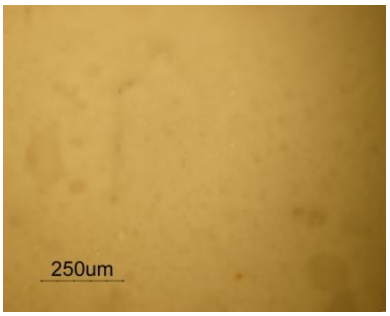


30sec.,100 x

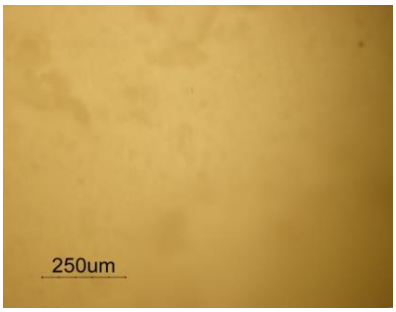
1min., $50x$



1min., 100 x

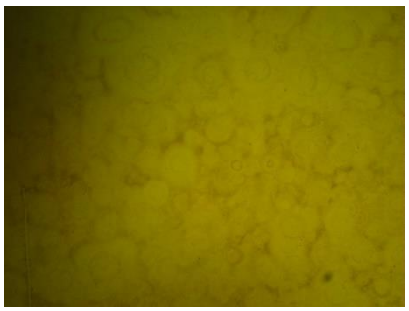


1.5min., 50x 1.5min., 100 x

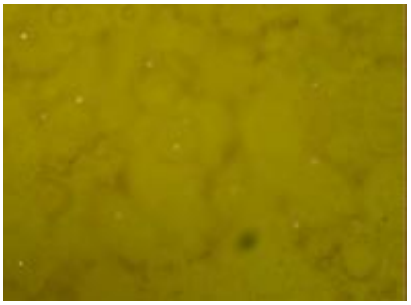


2min.,50x 2min.,100x

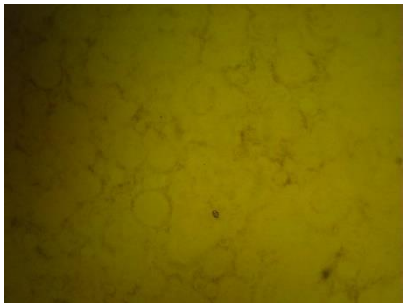
(Figure3.1): Optical micrograph (50x, 100x) view of cpTi coated with nano HA for different times.



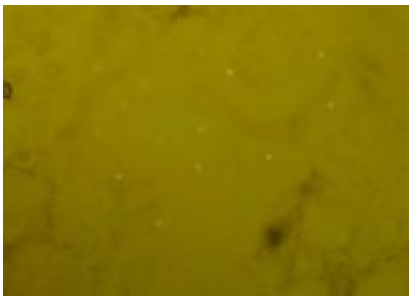
0.5min., 50 x



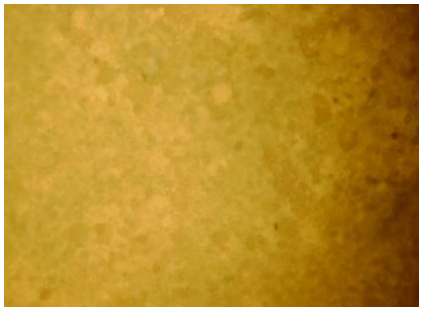
0.5min., 100 x



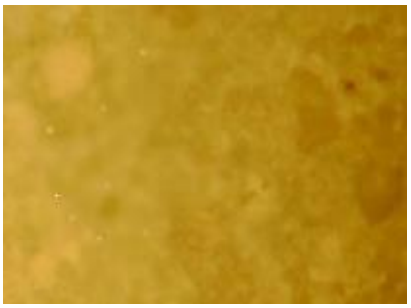
1min, .50 x



1min., 100 x



1.5min., 50 x

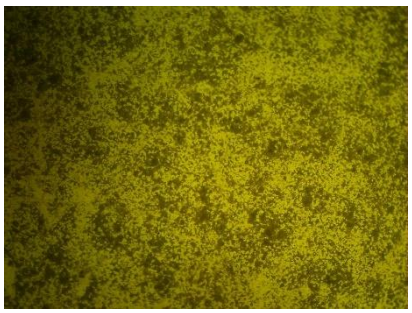


1.5min., 100 x

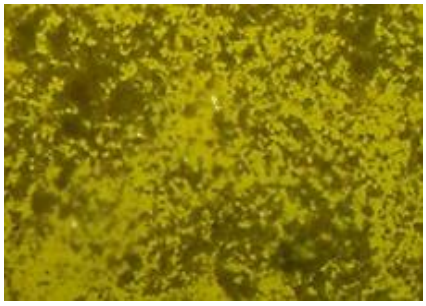
2min., 50 x

2min., 100 x

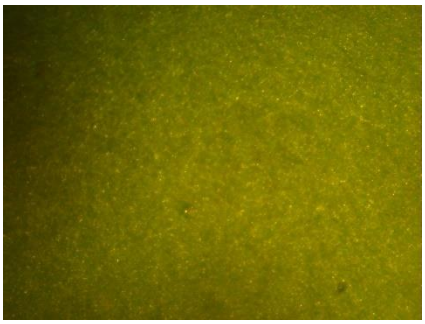
(Figure3.2): Optical micrograph (50x, 100x) view of cpTi coated with nano HA -chitosan mixture for different times



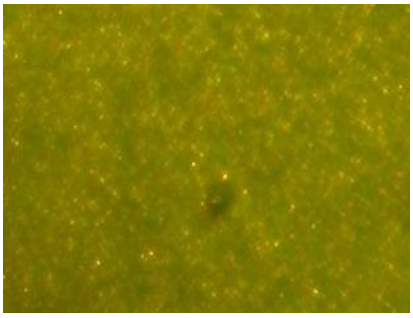
0.5min., 50 x



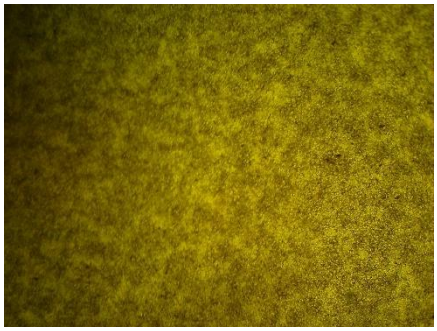
0.5min., 100 x



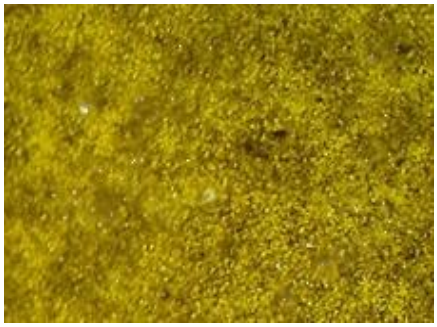
1min., 50 x



1min., 50 x



1.5min., 50 x



1.5min., 100 x

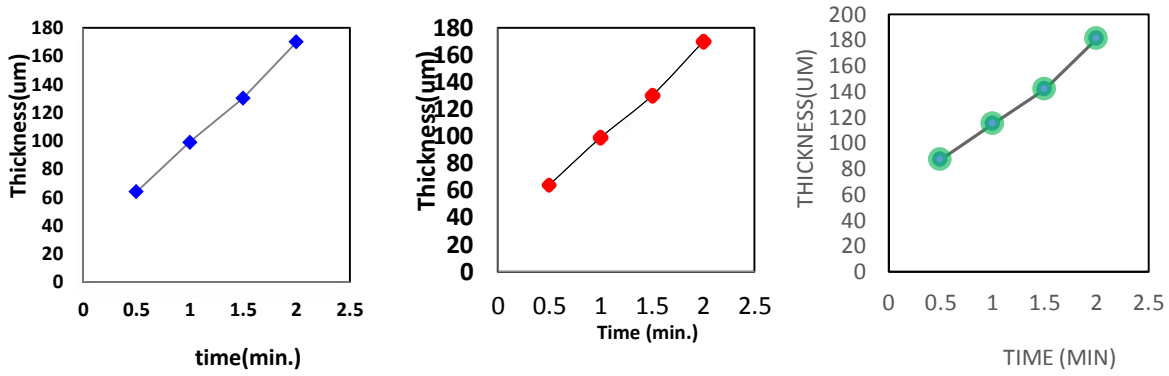
2min., 50 x

2min., 100 x

(Figure3.3): Optical micrograph (50x, 100x) view of cpTi coated with nano HA chitosan and collagen mixture for different times

3.1.2 Measurement of coating layers thickness

The thickness of the coated layers was measured by the Erichsen mini test micro process thickness gauge. The coating thickness of the coated film were increased with the increasing of the coating time (Figure 3.4)



(A) *(B)* *(C)*

(Figure3.4): The relation of coating film thickness (µm) with time of deposition (min) (A) NanoHA coating layer (B) NanoHA-chitosan coating layer(C) Nano HA,chitosan &collagen coating layer

3.1.3 X-ray Diffraction of coated samples

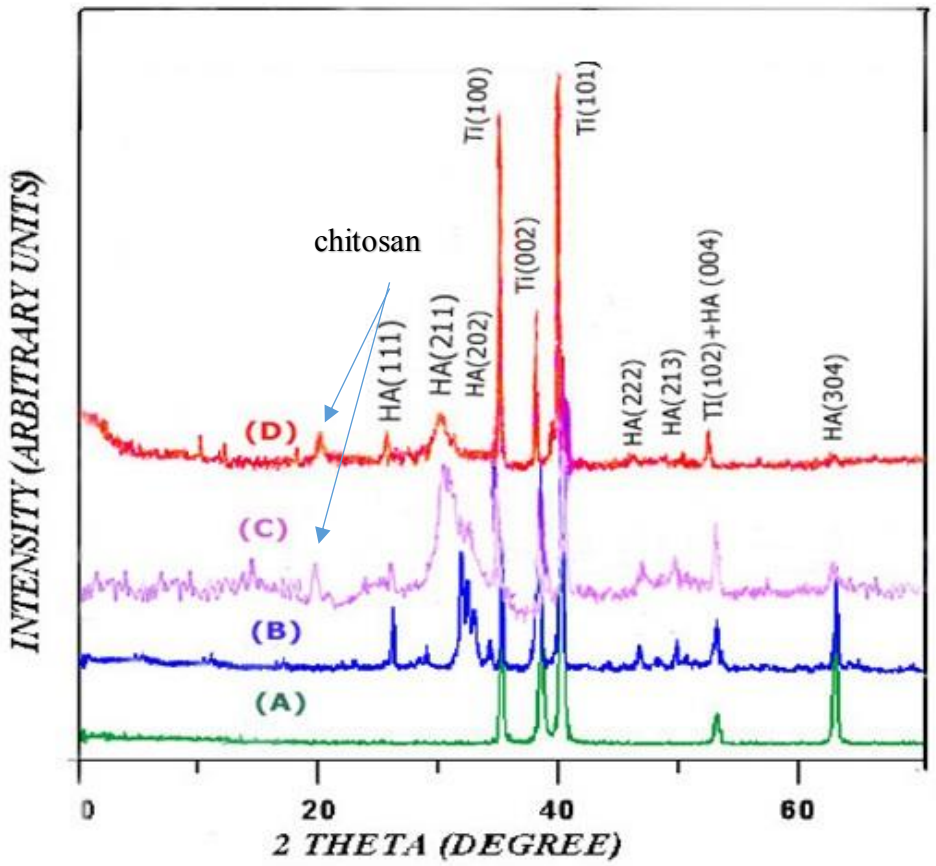
Phase analysis of nano HA, nano HA-chitosan and nanoHA-chitosan-collagen coatings were studied using X-ray diffractometer as shown in (***Figure 3.5).*** The 2Θ angles were swept from 0-70° in step of one degree. The peak indexing was carried out based on the JCPDS (joint committee on powder diffraction standards) International Centre for Diffraction Data, ICDD file # 44.1294 for titanium, # 90.432 for HA.

(A)The strongest lines of this phase were at (100), (002), (101) and (102) at 2Θ 35.6775, 38.8071, 40.7429 and 53.5361, for uncoated CpTi discs.

(B) The pattern show strong peak at (002), (210) (211), (202), (222), (213), (004) and (304) at 2Ɵ 26.0632, 29.5585, 31.9910, 34.2461, 46.9280, 49.5284, 53.7266 and 63.6232for nano HA coated discs.

(C) Xrd pattern of nano HA-chitosan mixture show in addition to peaks of nano HA, it show peak at 2^{θ}:20.1826 this may reflect peak of chitosan.

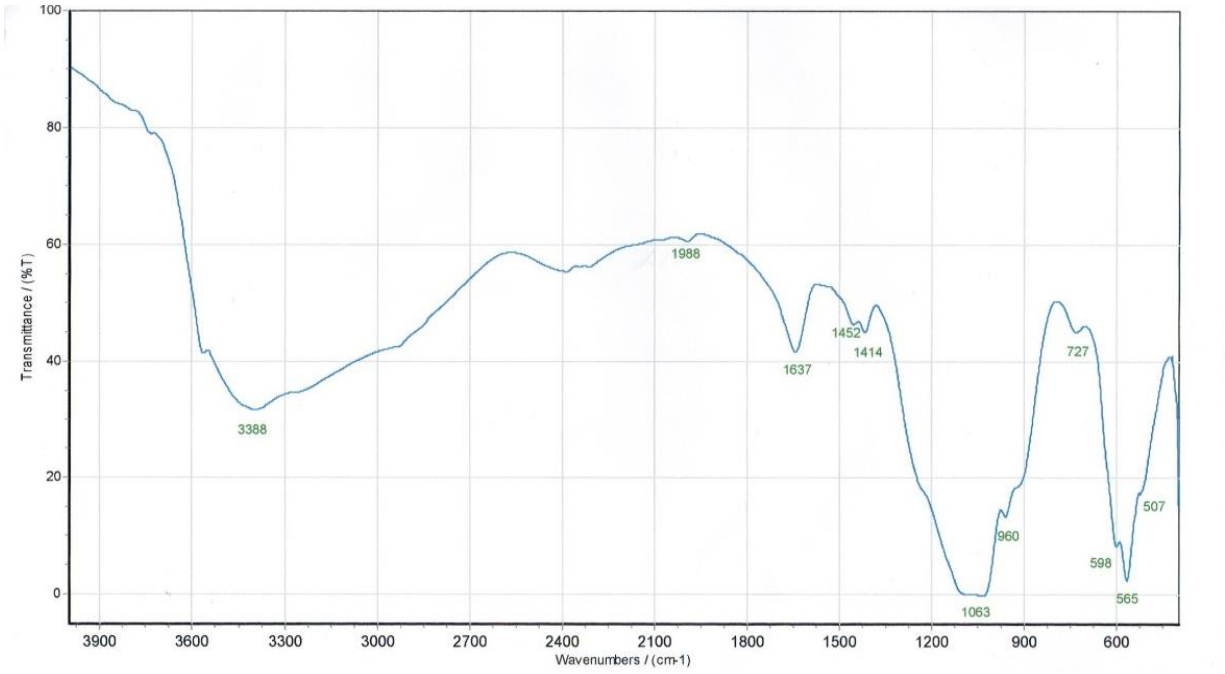
(D) Xrd pattern of nano HA-chitosan-collagen composite show no additional peak appeared with decrease in intensity of HA and chitosan peaks.



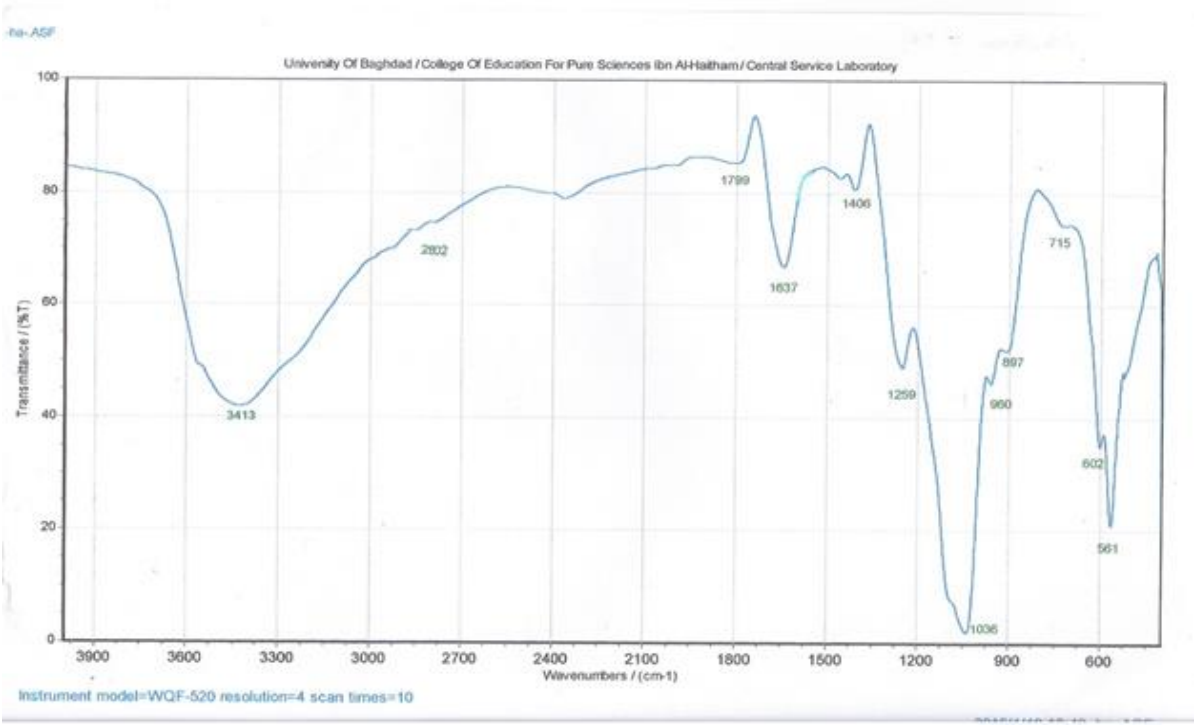
(Figure3.5): X-ray diffraction patterns (A) uncoated Ti (B) Ti substrate coated with nano HA (C) Ti substrate coated with nano HA-chitosan composite (D) Ti substrate coated with nanoHA-chitosan-collagen mixture

3.1.4 FTIR analysis

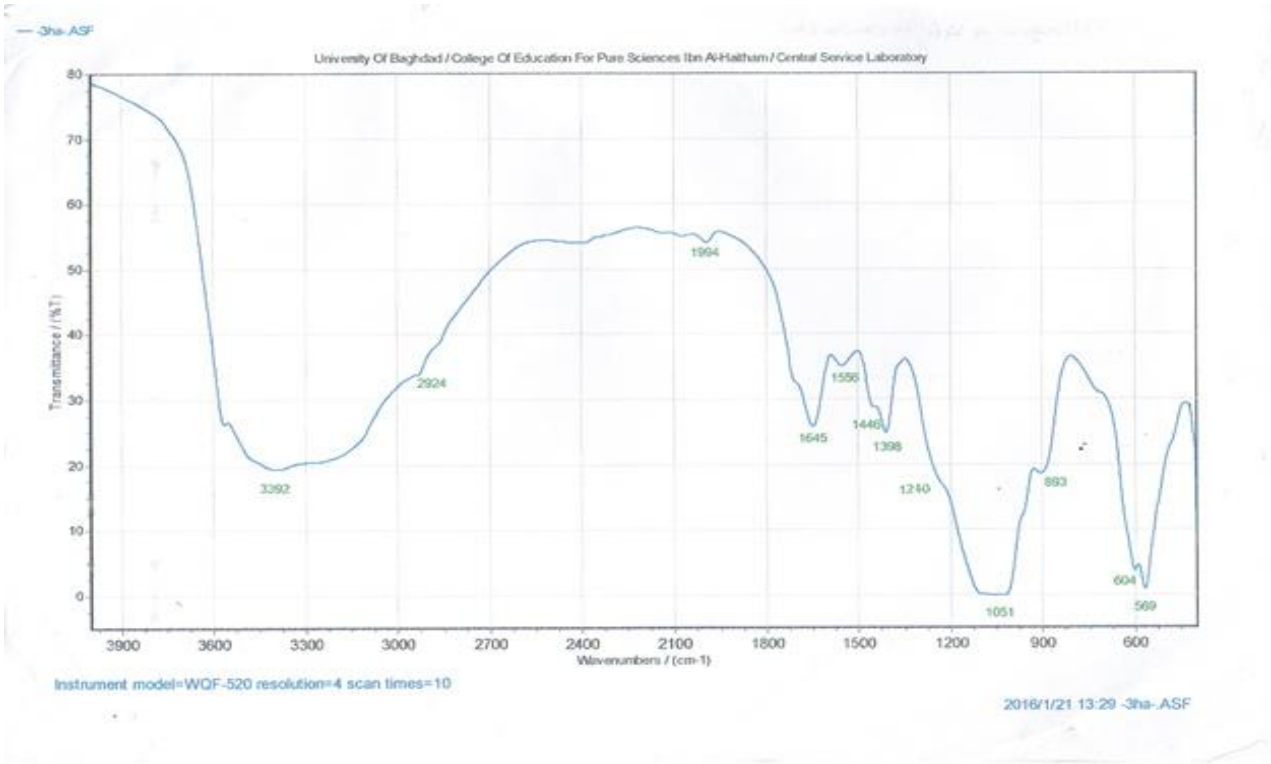
Spectra for nano HA, mixture of nano HA-chitosan and nano HA –chitosan –collagen coating are showed in (***Figure 3.6)***, (***Figure 3.7)*** and (***Figure 3.8)*** respectively. Spectra for nano HA coating(figure3.4) showed the characteristic vibration peaks; at 3388 for stretching of OH-,960-1063 stretching of PO_4^{-3} ,1630 bending(OH-),565,598bending of PO_4^{-3} ,1414 1452stretching (C-O-C). While spectra of mixture of nano HA –chitosan (figure 3.5) showed shifting of peaks of PO_4group in HA to 561; 602, the band of (C-O-C) of HA shifted to1406, the bands at 1637 and 1799correspond to OH group of HA and amide I and amide II of chitosan, while 2802assigned to hydroxyl group of chitosan, band at 1259 correspond to C-O group of chitosan. Spectra of mixture of nano HA-chitosan and collagen composite coating(figure 3.6) showed band at 1398 indicated to amide III of collagen, 1554 and 1654 assigned to amide I and II of collagen,1446 correspond to (C-O-C) of HA,2924assigned to hydroxyl group of chitosan, band of C-O group of chitosan shifted to 1240.



(Figure3.6): FTIR analysis of nano HA coating



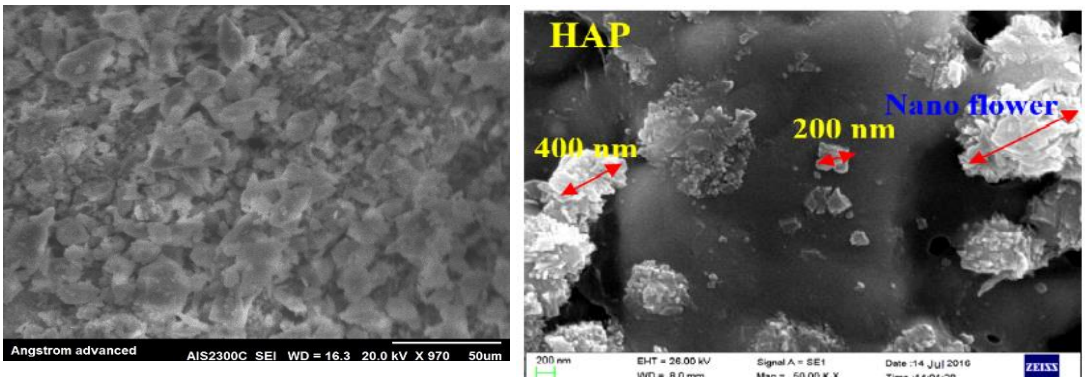
(Figure3.7): FTIR pattern of nano HA-chitosan composite



(Figure3.8): FTIR pattern of nano HA-chitosan –collagen composite

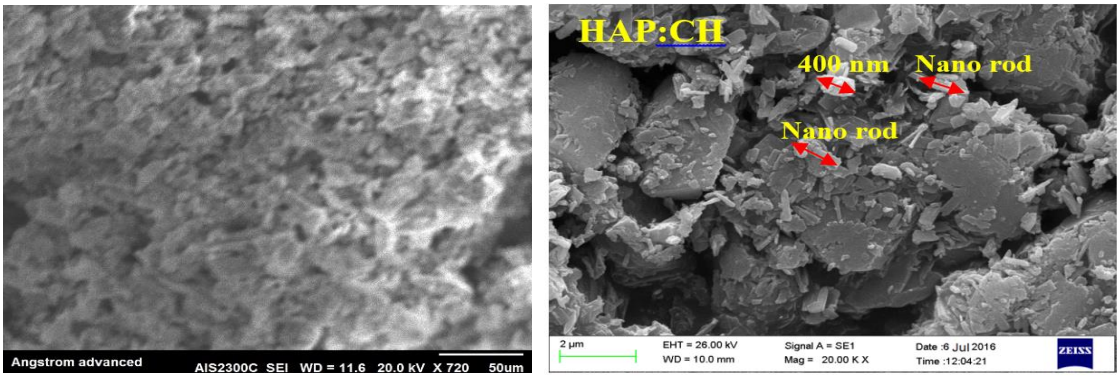
3.1.5 Nanosurface feature [Morphological analysis (SEM)]

SEM micrographs of cpTi plate coated with nano HA (***Figure 3.9)***, and mixture of nano HA and chitosan (***Figure 3.10),*** and mixture of nano HA chitosan and collagen (***Figure3.11).*** In these figures, the changes in the surface were observed at low and high magnification. In the SEM micrograph of coated discs, there are many irregular projections, and the picture of the surface had a feature or a structure nano flower (seen in nanoHA coating) and nano rods (in nano HA and chitosan composite coating), and nano particle (seen in nano HA, chitosan and collagen composite coating).with particle size of about 400nm.



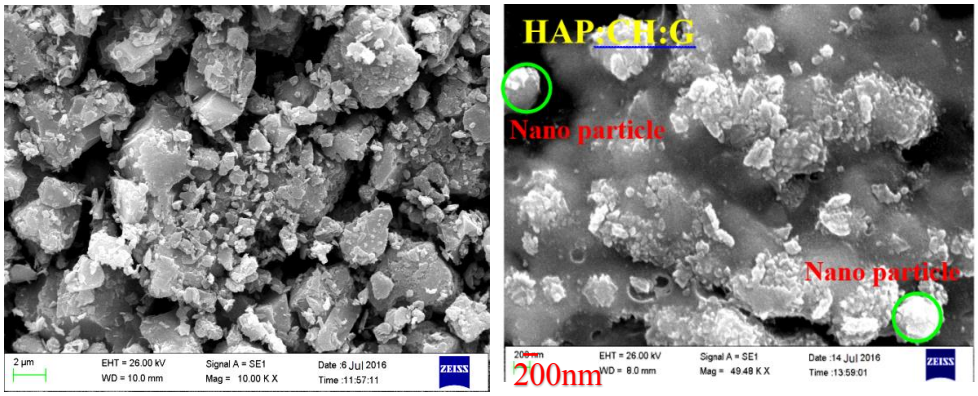
(A) 50µm **(B) 200nm**

(Figure3.9): SEM analysis of nano HA coating



(A) 50 µm **(B) 2 µm**

(Figure3.10): SEM pattern of nano HA-chitosan coating.



(A) 2 µm **(B) 200nm**

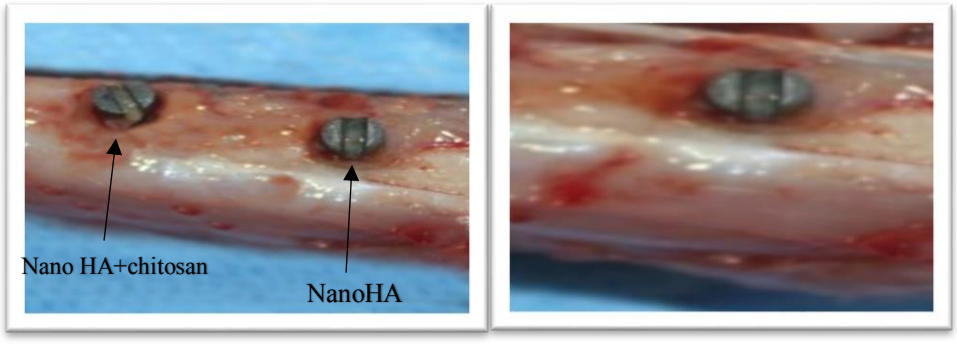
(Figure3.11): SEM pattern of nanoHA-chitosan-collagen composite

3.2 In vivo Experiments:

3.2.1 Clinical findings

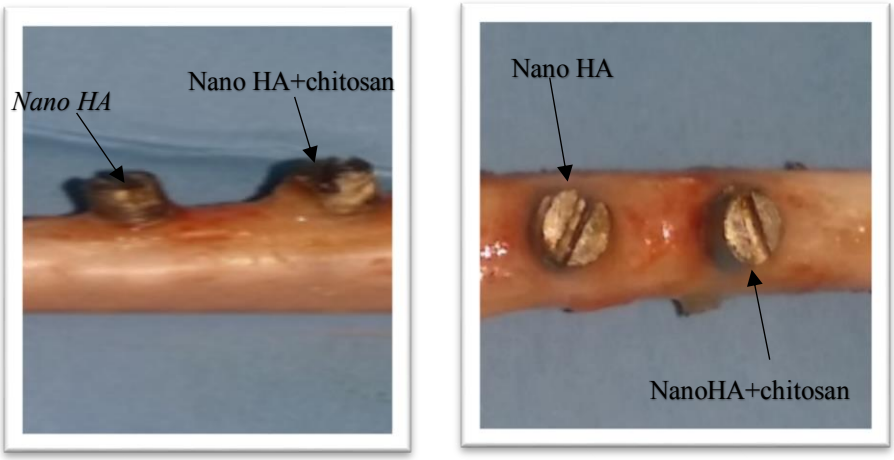
All animals recovered well after surgery and they showed normal movement after one week which indicates that they tolerated the implantation.

At sacrifice, no sign of gross infection was observed around the implant in surgical sites. All implants were found stable in the bone, they could not be moved with manual force after 2 and 6 weeks of healing periods because of formation of new bone (**Figure 3.12), (Figure 3.13), (Figure 3.14), (Figure 3.15).**

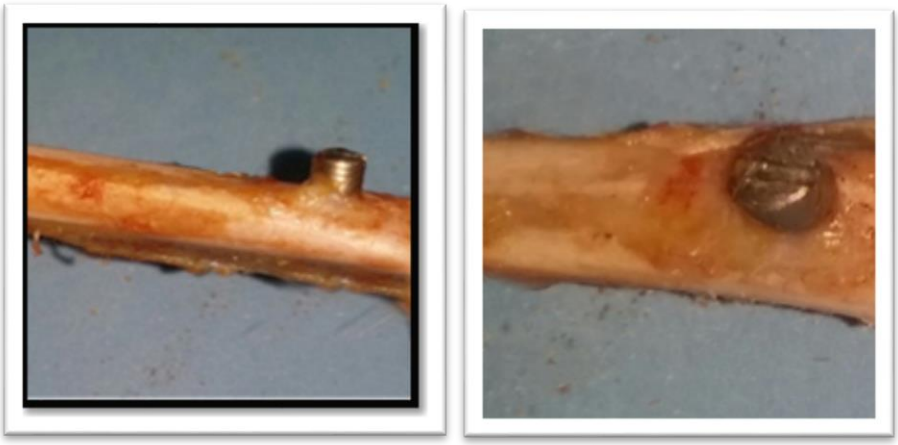


(A) Right tibia ***(B) left tibia***

(Figure 3.12): surgical site after 2weeks

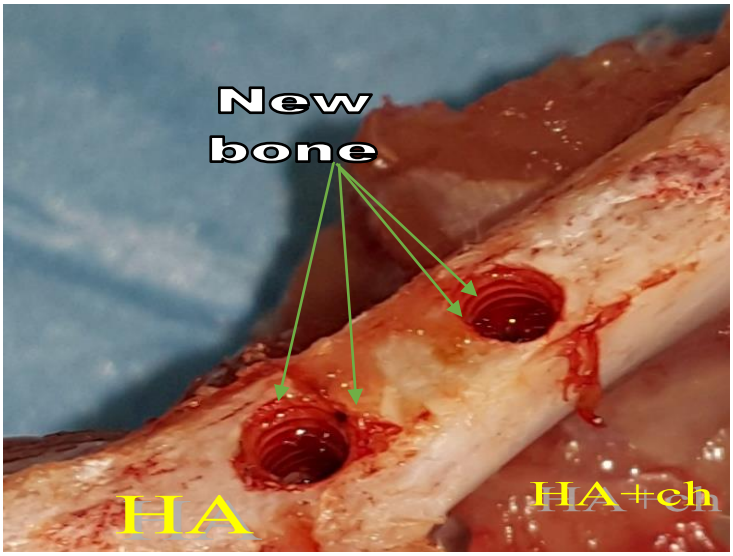


(A) Right tibia, Lateral view *(B) right tibia, Top view*

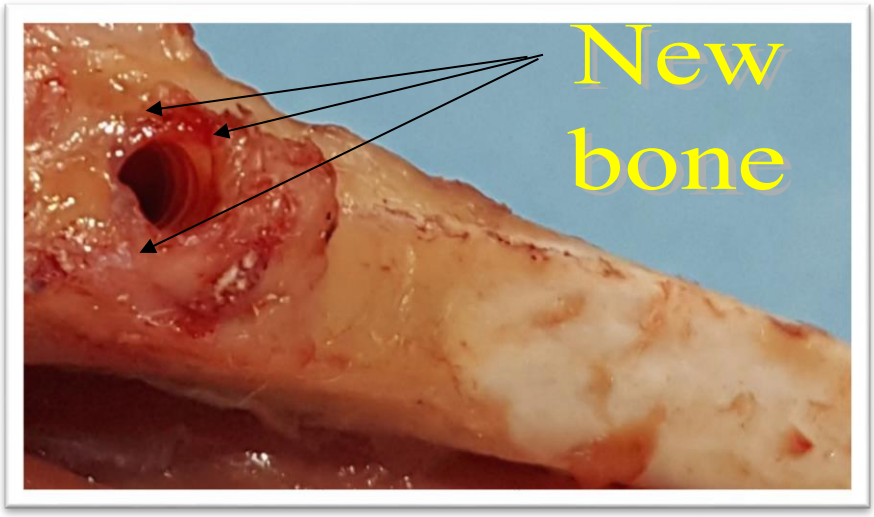


(A) Left tibia, Lateral view *(B) Left tibia, Top view*

(Figure3.13): surgical site after 6weeks



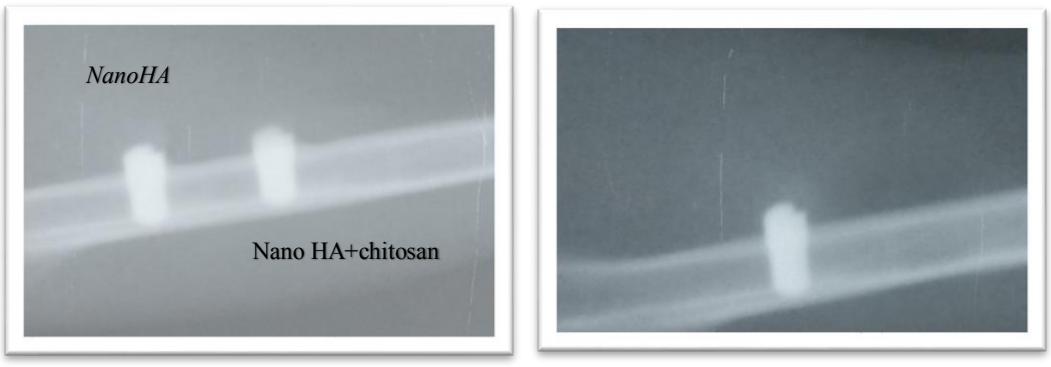
(Figure3.14): surgical site of right tibia after removal of screws after 6weeks show new bone formed between threads



(Figure3.15): left tibia after removal of screw after 6weeks, showing new bone formation

3.2.2 Radiographic evaluations

The results of radiographic evaluation showed that there were no areas of radiolucency between the nano coated implant and adjacent cortical bone in the radiographic examination and also there was an increase in the thickness of cortical bone around the implants after 6 weeks healing periods as shown in (**figure3.16)**



(Figure3.16): Radiographic view showed nano coated implants 6 weeks post implantation

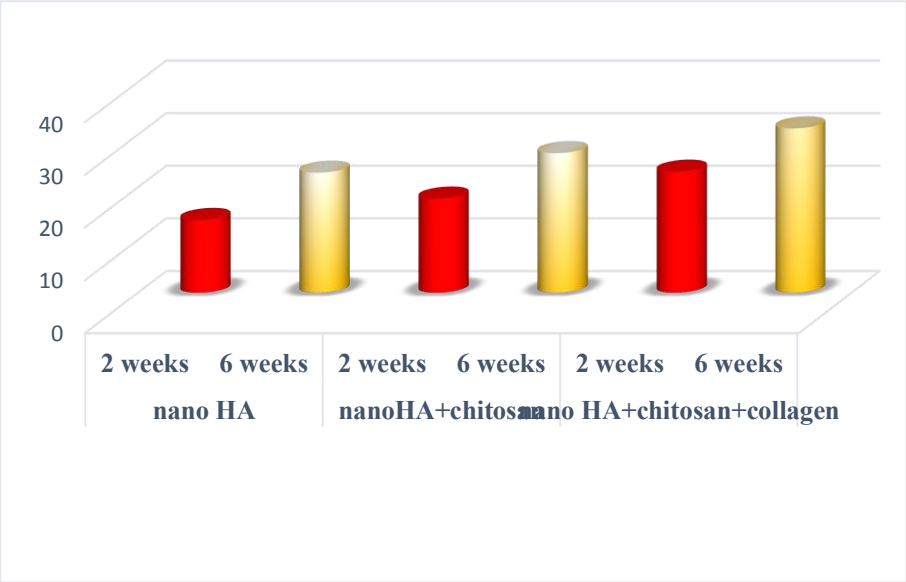
3.2.3 Mechanical testing

Table 3.1 and **(figure 3.17**) show descriptive statistics of removal torque values of cpTi screws coated at both time intervals. After 2 weeks of implantation, the mean value was needed to remove the implants coated with nano HA only (13.76 N.cm), for the mixture of nano HA-chitosan coating, the mean value was (17.82N.cm). While the highest mean value which needed to remove screws was seen in nano HA-chitosan-collagen composite was (22.945N.cm).

After 6 weeks of implantation, the mean value was needed to remove the implants coated with nano HA only (22.76N.cm), while with a mixture of nano HA-chitosan coating, the mean value was (26.47N.cm). And the highest mean value which needed to remove screws was seen in nano HA-chitosan-collagen composite was (31.18N.cm).

Table (3.1):- descriptive analysis of torque value of nano HA, mixture of nano HA-chitosan and mixture of nano HA-chitosan and collagen coated groups at 2 and 6 weeks healing periods.

group	Time (weeks)	no.	mean	S.D	S.E	MIN.	Max.
nano HA	2	6	13.76	2.39446	0.97753	10.59	17.65
	6	6	22.76	4.126	1.684	17.65	28.24
nanoHA+chitosan	2	6	17.82	2.8822	1.1767	14.12	21.18
	6	6	26.47	2.95	1.205	24.71	31.77
nano HA+chitosan+collagen	2	6	22.945	4.8658	1.9864	17.65	28.24
	6	6	31.18	2.657	1.084	28.24	35.31



(Figure 3.17): A Bar chart showed the summary of the differences in the torque mean values between all groups

The equality of means between all groups of implants interval tested by ANOVA test demonstrated highly significant difference at P≤0.01 with 2 degrees of freedom as shown in **Table (3.2).**From this table it appeared that there is highly significant differences in torque mean value between 3 groups of implant in each healing interval.

Table (3.2): Equality of means of removal torque value by ANOVA for all three groups of implants at 2 and 6 weeks intervals independently.

Time interval	Sum of Squares (S.S)			d.f	Mean of Squares M.S		F-test	Sig.
	Within groups	Between groups	Total		A.	B.		
2weeks	253.612	188.582	442.14	2	126.80	12.57	10.086	0.002 HS.
6weeks	265.833	164.069	429.90	2	132.91	10.93	12.152	0.001 HS.

For the multiple comparisons, the least significant difference (LSD) test used for equality of torque mean values among different groups after two weeks and six weeks healing periods. (**Tab 3.3)** showed a significant difference between groups except coating with mixture of nano HA, chitosan and collagen composite coating 2 weeks in comparison with coating of implants with nanoHA in 2 weeks and coating with mixture of nanoHA chitosan and collagen in 6weeks in comparison with coating nano HA in 6weeks show highly significant difference.

Table (3.3) Multiple Comparison (LSD) among all pairs of different periods of healing times in each group of cpTi implant screws independently

Groups		*Mean Difference*	*Sig.*
HA (2weeks)	HA+ch. (2weeks)	-5.06000*	.016
	HA+ch.+Coll (2weeks)	-9.17833*	.000
HA+ch. (2weeks)	HA+ch.+Coll (2weeks)	-4.11833*	.046
HA(6weeks)	HA+ch. (6weeks)	-4.70607*	.04
	HA+ch.+Coll (6weeks)	-9.41333*	.000
HA+ch. (6weeks)	HA+ch.+Coll (6weeks)	-4.70667*	.024

The mean difference is significant at the 0.05 level.

The mean difference is highly significant at the 0.01 level.

T-test was performed for testing the equality of means presented a highly significant differences at $P<0.01$ between all groups of coating at two periods of time, table (3.4).

(Table 3.4) t-test for equality of means of torque value for three groups of coating at 2 and 6 weeks intervals.

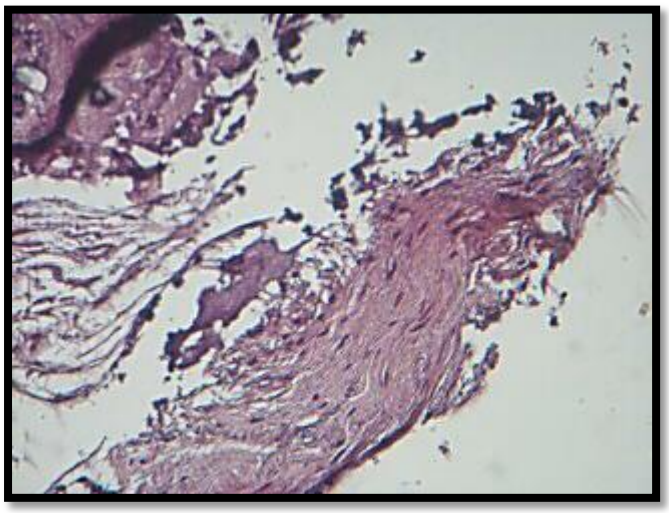
Group of coating	***Time***	***Mean Difference***	***t-test for Equality of Means***			
			t	***df***	***P value***	***Sig.***
Nano HA coating	***2×6 weeks***	***8.001***	***-4.108***	***10***	***0.002***	***HS.***
Nano HA,chitosan coating	***2×6 weeks***	***7.648***	***-4.450***	***10***	***0.001***	***HS.***
Nano HA, chitosan and collagen coating	***2×6 weeks***	***8.236***	***-3.639***	***10***	***0.005***	***HS.***

3.2.4 Histological features of implant after 2 and 6 weeks healing periods.

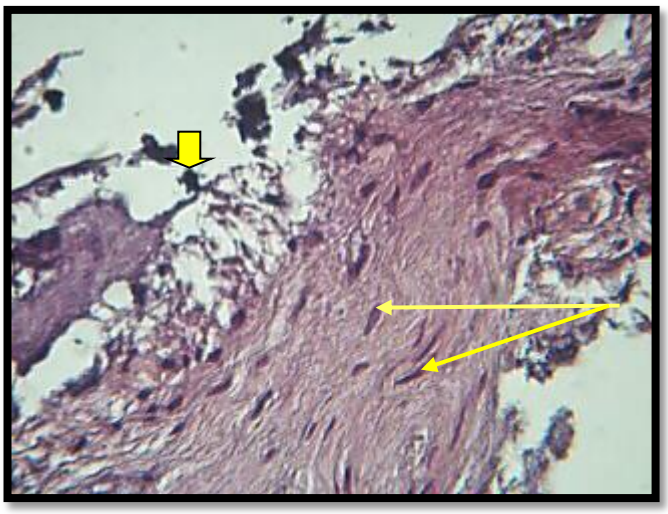
1- Two weeks after implantation

A. Nano HA coated cp Ti implants

Histological view of nano HA coated screw implanted in rabbit tibia after 2 weeks illustrates thread area filled with woven bone, fibrous connective tissue, fibroblasts, new blood vessels and osteoprogenitor cells ***(figure 3.18).***



(*A):Histological view of nano HA Coating after 2 weeks shows thread area filled with woven bone, fibrous connective tissue., fibroblasts, and blood vessels, H&E stain X20.*

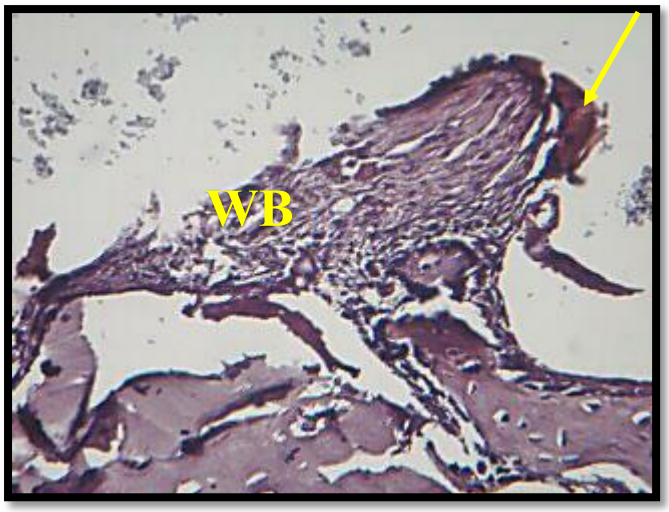


(B): Higher magnification view shows woven bone, fibrous connective tissue. Fibroblasts (arrows), and osteoprogenitor cells (arrow head), H&E stain X40.

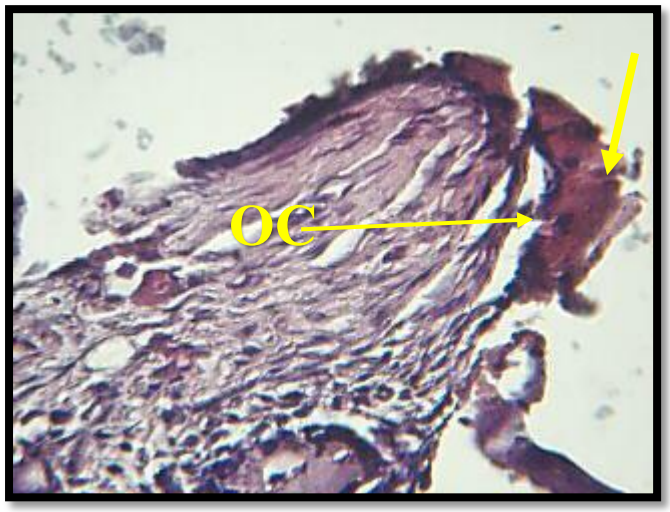
(Figure3.18 A, B) Microphotographic view of sections occupied by nanoHA coated screws after 2 weeks of implantation in rabbit tibia.

B. Nano HA-chitosan mixture coated cp Ti

After 2 weeks of implantation of screw coated with nanoHA-chitosan mixture, its histological view shows thread area filled with Woven bone, bone spicules with osteocyte cells ***(figure 3.19).***



(A): Histological view of nanoHA-chitosan mixture after 2 weeks shows thread area filled with Woven bone (WB) and bone spicules (arrow), H&E stain X20.

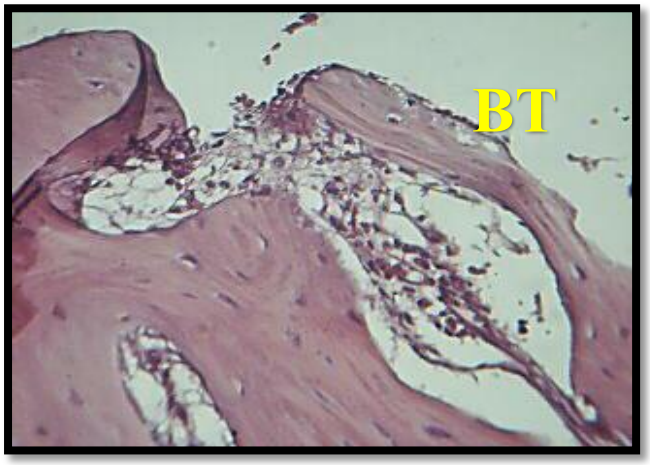


(B): Magnifying view shows woven bone with osteocytes (OC) and bone spicule (arrow), H&E stain X40.

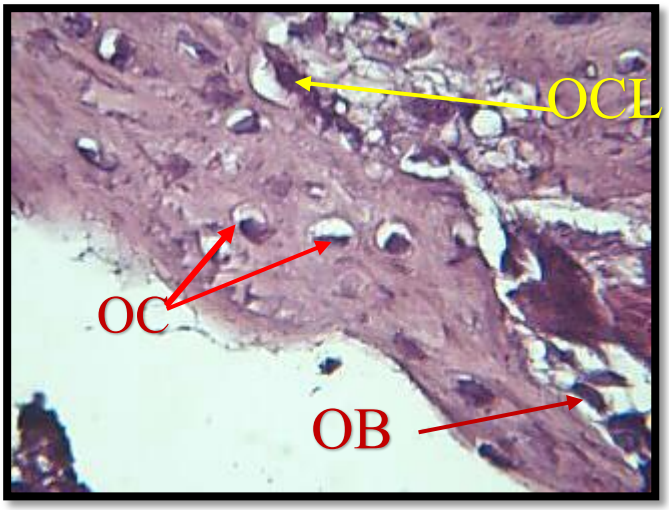
(Figure 3.19 A, B) Microphotographic view of sections occupied by nanoHA-Chitosan coated screws after 2 weeks of implantation

C. Nano HA-chitosan-collagen mixture coated cp Ti

Histological view of nanoHA-chitosan-collagen mixture coated screws after two weeks of implantation, shows thread area filled with new bone trabeculae filled with large size osteocytes surrounded by osteoblast and osteoclast cells **(Figure3.20).**



(A): Histological view of nano HA-chitosan-collagen mixture after 2 weeks shows thread area filled with new bone trabeculae (BT), H&E stain X20.



(B): Magnifying view shows new bone trabeculae filled with osteocytes (OC) and surrounded by osteoblasts (OB) and osteoclasts (OCL), H&E stain X40.

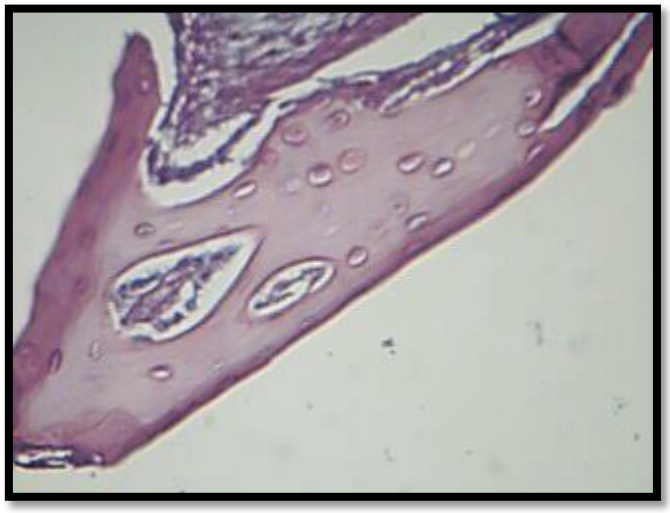
(Figure 3.20 A, B) Microphotographic view of sections occupied by nanoHA-Chitosan-Collagen coated screws after 2 weeks of implantation

2- Six weeks after implantation:

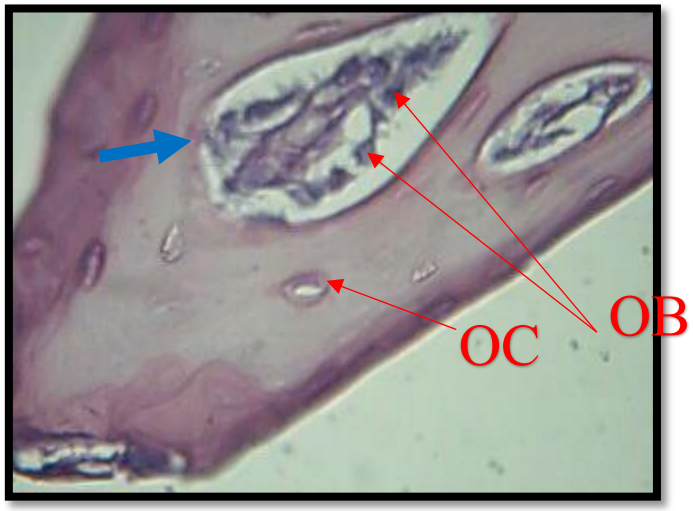
A. Nano HA coated cp Ti

The histological view of nanoHA coated cp Ti screws implanted in the rabbit tibia after six weeks shows the thread site filled with new bone, and

at the higher magnifying view shows that new bone filled with osteocyte cells and osteoblast lining the harversian canal (***figure3.21***).



A): Histological view of nano HA after six weeks shows thread area filled with new bone formation, H&E stain X20



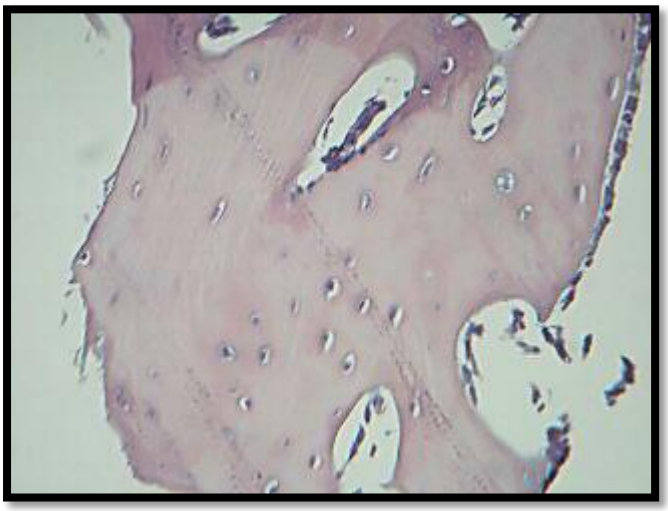
(B): Magnifying view shows new bone filled with osteocytes (OC) and Harversian canal(Blue arrow) lined by osteoblasts (OB), H&E stain X40.

(Figure 3.21 A, B) Microphotographic view of sections occupied by nanoHA coated screws after 6 weeks of implantation

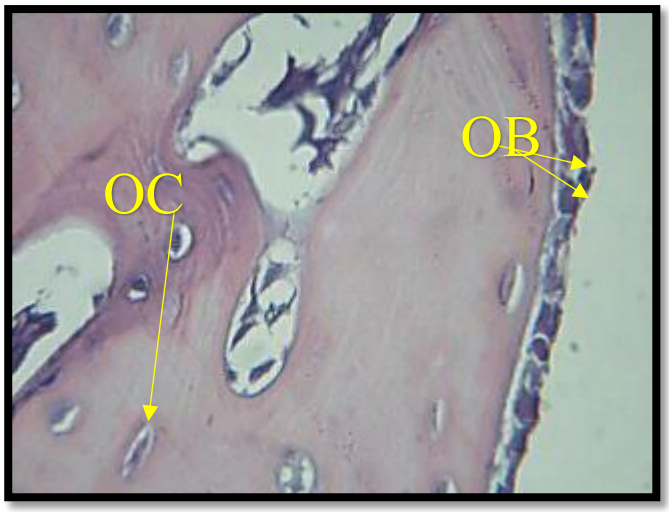
B. Nano HA and chitosan mixture coated cp Ti

The histological view of nano HA-chitosan mixture coated cpTi screws after six weeks of implantation show formation of dense new bone,

at higher magnifying power the dense new bone show large size osteocyte cells and lined by single row of osteoblast cells ***(figure3.22)***



(A): Histological view of nano HA-chitosan mixture coating shows thread area filled with dense bone formation, H&E stain X20.



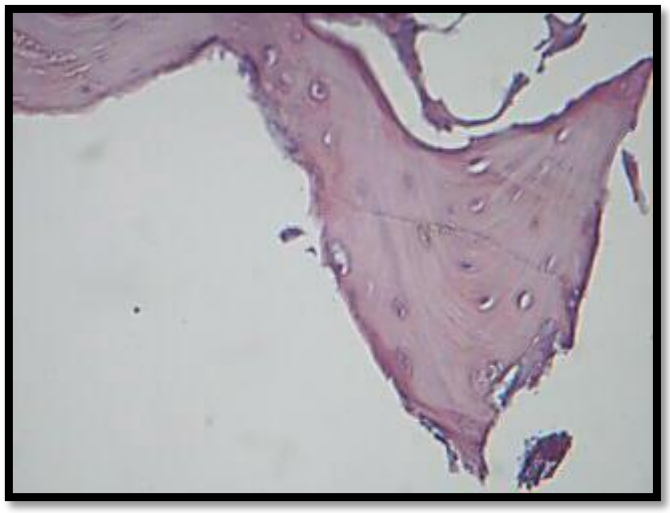
(B): Magnifying view of shows dense bone filled with large size osteocytes (OC) and lined by single row of osteoblasts (OB), H&E stain X40.

(Figure 3.22 A, B) Microphotographic view of sections occupied by nanoHA-Chitosan coated screws after 6 weeks of implantation.

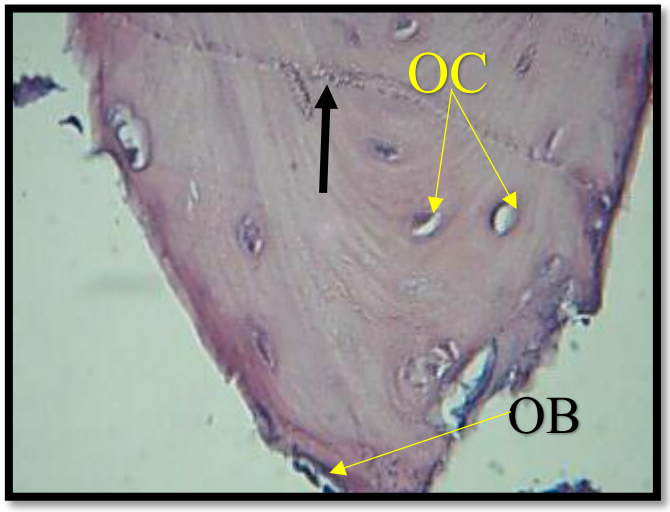
C. Nano HA, chitosan and collagen coated cp Ti:

The histological view of nanoHA-chitosan-collagen mixture coated cp Ti screws after six weeks of implantation shows formation of mature bone

at the thread area, at higher magnifying view shows mature bone filled with lamellar arrangement of osteocytes and lined by osteoblasts (**figure3.25).**



(A): Histological view of nanoHA-chitosan-collagen mixture shows thread area filled with mature bone, H&E stain X20.



(B): Magnifying view of previous picture shows mature bone filled with lamellar arrangement of osteocytes (OC) and lined by osteoblasts (OB) reversal line (black arrow), H&E stain X40.

(Figure 3.23 A, B) Microphotographic view of sections occupied by nanoHA-Chitosan-Collagen coated screws after 6 weeks of implantation

Chapter Four

Discussion

Chapter Four

Discussion

Implant osseointegration affected by surface characteristics of implants, like micro- and nano-roughness and chemical composition of implants **(*Mendonca et al., 2009)***.

Because of high biocompatibility, osteoconduction and chemical similarity of hydroxyapatite with human bone and teeth, so hydroxyapatite has been considered as a promising biomaterial ***(Sharma et al, 2009).***

Chitosan and collagen are polymeric materials. They are widely used in the biomedical field, because of their similarity to the physical-chemical properties of the living tissues, could improve the bioactivity and the bone bonding ability. Reducing potential damage to soft tissue in the site of the implant and enhancing mechanical strength can be achieved by calcium phosphate with biopolymers composite. ***(Carlos et al., 2010)***.

4.1 In vitro experiment:

4.1.1 Dip-Coating

Dip-coating (Sol-gel process) has a number of advantages over other coating processes such as flexibility, control of coating morphology. This method require simpler equipment and is of a lower cost. (P_2O_5) is an additives applied in this method, used as a thickening material, ***(Aksakal and Hanyalogla, 2008).***

The sol–gel method is extensively used to deposit thin (<200 μm) coatings. Coating with thin biocompatible films can enhance osseointegration of the metallic implants.. Dip-coating method results in a homogeneous and pure coating also the lower processing temperature avoids the phase transition. ***(Liu et al., 2004).***

Improving the bone implants properties by the coating of metallic implants with thin biocompatible films having good mechanical properties in order to enhance the osseointegration of these implants, ***(Heness and Ben-Nissan, 2004)***.

The main problem of metallic implants coatings is their poor adhesion to the metallic objects because of thermal coefficients difference between them, this causes the coatings obtained by higher temperature to fail either during the mounting procedure, or during service in the human body . If the coating film is too thick the coherent force within the film will result in a film that shrinks in a parallel direction to the substrate surface, cracking may result if this coherent force is too high when heated later to drive out the remaining solvent and water,. in thin film coating the adhesion force prevents the shrinkage in the direction parallel to the surface, so the films shrinks perpendicular to the surface, resulting in a film that is strongly attached to the surface***(Mellor,2001).***

The dip-coating method and the electro deposition process are considered as the best processes of obtaining these ceramic coatings *(**Ghiban et al., 2006**)*.

In the present study, dip-coating technique showed that the coating thickness increased with deposition time. The dip coating showed a thin continuous, uniform thickness layer of coating material, as agreement with ***(Hussein, 2015)*** although the materials used are different.

4.1.2 Optical microscopical observations of coated samples

A smooth and homogeneous coating films have been obtained by dipping and after sintering under continuous flowing of argon gas and that agree with **(*Aksakal and Hanyalogla, 2008)*.** In this study cracks were not observed across the coating layer deposited by dipping technique for all coating materials and this is due to the controllable way of deposition of the materials and sintering under low temperature also control the amount of P_2O_5 (0.01g in 50 ml of ethanol) this agree with work of ***(Hussein, 2015)*** and not agree with the work of

(***Jamil, 2011***) who proved the presence of crack in the coated layer in optical microscopical examination, with the difference in the coating materials and techniques used other than these in present study.

4.1.3 XRD phase analysis

It is evident from the XRD patterns the specimen coated with nanoHA was well covered because most of the diffraction lines were indexed to nano HA corresponding to JCPDS file 90.432.for the specimen coated with mixture of nanoHA-chitosan it show in addition to peaks of nanoHA, it show peak at 2^{θ}:20.1826 with differences in material, method technique, and the shape of implant used in this study this agree with (***Ajay et al., 2011)***.

The difference in XRD pattern could be due to interactions between ceramic material and polymeric material.

The presence of Ti peaks in the XRD pattern after coating process could be due to the penetration of X- rays beyond the coated layer(***Jani ,2014).***

As XRD shows that the narrower peaks are indicative of layer consists of highly crystalline form, whereas broad peaks represent lower levels of crystallinity, this come in agreement with **(*Kweh et al., 1999)*** with the difference in the coating materials and techniques used.

4.1.4 FTIR Analysis

The result of FTIR spectra for nanoHA, mixture of nanoHA-chitosan and mixture of nanoHA, chitosan and collagen recorded changes as the shifting of some vibration peaks and change in appearance, this shifting together with the relative change in intensity suggested the occurrence of molecular interactions between the side groups or end groups of mixed materials ***(Wang et al. 2007***).

This could be due to interaction between nanoHA with chitosan, and interaction of collagen with nanoHA and chitosan. This interaction could be explain the difference in properties of coating layer among groups.

4.1.5 Surface morphology (scanning electron microscope)

Results provided from the scanning electron microscope revealed that no inorganic aggregate could be observed, indicating that the inorganic phase is well dispersed in the organic Col-Chi system without phase separation and that the size of the inorganic particles is very small, agrees with (***Wang et al. 2007***).

4.2 In vivo experiments

4.2.1 Experimental animals

Adult male New Zealand white rabbits were selected because they have many appropriate properties to fit the requirement of the study. Many implant investigations have been successfully used the rabbit tibia as implantation site ***(Al-Mudarris, 2006; Hammad, 2007; Salman, 2011; Jamil, 2011 and Shukur, 2014; Jani, 2014; Hussein, 2015 and Refaat, 2016)***.

Rabbits reaches skeletal maturity shortly after sexual maturity around 6 months of age. Because the remodeling of rabbit cortical bone is faster when compared with human beings, it permits for evaluation of osseointegration of dental implants as early as 6 weeks compared with 18 weeks in human.The age of the animals used in this study was about 11 months thus assuring complete closure of proximal tibia epiphysis ***(Pearce et al., 2007).***

Because the dimensions of tibia of rabbits correspond well with human alveolar space, the tibia were chosen to simulate the clinical situation, also tibia bone provides easy access by Ti implant.

The rabbit tibia bone morphology permit the Ti screw to engage bone cortex at coronal area and marrow apically (***Dahlin et al.,1989).***

4.2.2 Radiographical examination

The radiographic examination used for showing contact between bone and implant, radiolucent areas or any abnormal reaction to the implant. ***Atsumi et al., (2007),*** stated that it is difficult for a clinician to detect changes in the radiographic bone loss at 0.1mm resolution, so lack of radiolucency at implantation site is not an indication for osseointegration.

4.2.3 Mechanical test

4.2.3.1 Torque removal test

Torque defined as the twisting or movement occurred as a result of force acting at a distance on a body equal to the force multiplied by the perpendicular distance between the line of action of the force and the center of rotation at which it is exerted **(Hoda *et al.*, 2005)**.

Many investigations used torque removal test because it is considered an important parameter during studying and comparing screw shaped implants ***(Al-Mudarris, 2006; Hammad, 2007; Ostman et al., 2008; Faeda, 2009; Salman, 2011 and Jamil, 2011;Waheed, 2013 and Jani, 2014).***

4.2.3.2 Effect of coating materials: nano HA coating only and mixture (HA-chitosan and HA-chitosan-collagen) coating

The mixture of nano HA,chitosan and collagen coated cp Ti screws placed in rabbit bone recorded the highest mean of removal torque value at both healing period this indicated increased bond strength at the bone–implant area. HA coated implants tend to show osseointegration after implantation, because HA tend to dissolve partially rendering the surrounding fluids rich in calcium and phosphate ions and produce the precipitation of "bone-like" apatite on implant surface, encourage cellular differentiation and consequent bone formation, **(Al meida *et al.*, 2005).**

Chitosan stimulate the differentiation of osteoprogenitor cells so it can accelerate the bone formation and regeneration **(Kuma, et al., 2005).**

Collagen has direct role in biological functions of a cell (cell survival, proliferation and differentiation); it assists in healing of damaged tissues and bones (***Silvipriya et al., 2015).***

Due to different in particle size of HA used and different implant design, the mean torque value was recorded for HA coated cp Ti screws was also different, In this study the mean torque value was 13.76 N.cm after 2 weeks, whereas **Hussien, 2015** recorded mean torque value was 15.162 N.cm for the same period. .

The mean torque value for screws coated by mixture of nano HA-chitosan-collagen after 6 weeks of implantation was 31.18N.cm while for screws coated with nano HA-chitosan 26.47N.cm and 22.76 N.cm for nano HA coated screws record mean torque value after 6 weeks..

The higher amount of new mature bone formed in response to the mixture of nano HA,chitosan and collagen after 6 weeks, may reflect the higher bond strength at the implant bone interface and higher resistance to removal torque than the nano HA-chitosan composite and nanoHA only .

4.2.4 Histological analysis

Histological analysis is the most reliable method to assess stability of implants, and can be performed at any time of implantation (***Atsumi et al ,2007)***.

The results of present study showed that placing of coated implants within living bone resulted in osseointegrated implants under typical biological environment for bone formation, the rigidity of connection between osseointegrated implant and the bone and maturation of bone increases with time. This result agrees with ***(Liu et al., 2004; Waheed 2013)***.

Regardless of the type of coating material and the duration of the implantation the results gained revealed that no inflammatory reaction had happened during the experimental periods. This is in agreement with the results of ***(Yonus in 2000; Mano et al., 2002; Lins et al., 2003; Al-Mudarris 2006; Hammad, 2007;Waheed 2013; jani 2014).***

Histological feature of the nano HA coated implant in tibia bone after 2 weeks of implantation showed fibrous connective tissue and woven bone deposition, fibroblast, numerous capillaries and osteoprogenitor cells starting near the surface of the implant, this may indicate a delayed bone formation, these finding disagree with ***(Al-Ma'adhidi, 2002; Al-Najar 2009; Salman, 2011 and Hussein,2015)***.This disagreement may be due to the differences in material, method technique, together with the difference in shape of implant used in this study .

The woven bone formation began after the two weeks of implantation was seen in thread area of nano HA and chitosan coating. An osteoid tissue with numerous bone and osteoblast cells around the thread area with active blood vessels, which indicate the beginning of new bone formation. Regardless the difference in coating material and methods these findings are supported by the work of ***(Lins et al ,2003)***

The trabeculated thread was revealed in nano HA,chitosan and collagen composite coated dental implant after two weeks of healing interval, this is due to roughness in response to coating material which is of great importance in accelerating bone formation and this agree with ***(Refaat ,2016)*** who demonstrated that the tabeculated thread was seen in the nano coated dental implant.

The histological features of nano HA coated implants after six weeks show the thread site filled with new bone, which filled with osteocyte cells together with osteoblast lining the harversian canal, indicating that the deposition of bone was still processing. This findings agree with (***Hussein, 2015).***

After six weeks of implantation, new dense trabeculated bone as seen in thread area of nano HA, chitosan coating. Mature trabeculated bone, osteoblast surrounding the surface of the bone, osteocyte detected within bone matrix for nano HA, chitosan and collagen coating. This agrees with the (***Hussein, 2015),*** who demonstrated mature trabeculated bone was seen in the threaded area of implants coated with nanoHA with TiO_2 after six weeks of implantation**.**

The obtained results of this study showed that new bone was formed around coated implants without inflammatory reaction or fibrous encapsulation during the experimental periods regardless the type of implant coating and the duration of the implantation. Although the implants used in this study differ in material, technique, and shape of implant used, still the findings of this study agree with the findings of ***(Waheed 2013; Jani 2014; Hussein, 2015).***

The differences in the amount of bone tissue formed around coated dental implant in the cortical region and bone marrow were significant, suggesting a high osteoconductivity of the coating layer after addition of chitosan and collagen.

Finally one could concluded that rapid bone formation response to the coating are dependent on better biocompatibility of the material (nano HA ,chitosan and collagen) which greatly affects the histological and biomechanical properties of the bone implant interface with no sign of inflammation .

Chapter Five

Conclusion and Suggestions

Chapter Five

Conclusions and suggestions

5.1 Conclusions

From this study it can be concluded that:-

1. A composite coating of nano hydroxyapatite with chitosan-collagen by dip coating can be successfully synthesis, with homogenous, uniform thickness of coating and multifunctional surface has been created for proper osseointegration
2. There was a highly significant difference for the torque mean values after 2 and 6 weeks of implantation that resulted for all different groups.
3. Histologically, the use of a composite material(nano HA&chitosan and nano HA,chitosan &collagen) coated dental implant accelerate bone formation after 2 and 6 weeks implantation compared with that using nano HA alone.
4. This study indicated that the commercially pure titanium implants coated with (nano HA, nano HA and chitosan mixture and nano HA , chitosan and collagen mixture) are well tolerated by rabbit bone , revealed by the lack of inflammation and the high level of removal torque value due to bone formation

5.2 Suggestions for further studies:

1. Evaluate the mixing of nano HA with collagen coating materials on bone formation around dental implant.
2. Studying the effect of nano HA, chitosan & collagen composite and nanoHA & chitosan composite as a coating materials on other titanium alloy substrates like Ti 6Al 7Nb and Ti 6Al 4V.
3. Studying the effect of nano HA, chitosan & collagen composite and nanoHA & chitosan composite as a coating materials on cpTi using different coating methods.
4. Studying other properties such as adhesion, diffusion, wettability and roughness of the coated layer (nanoHA, chitosan &collagen composite and nanoHA, chitosan composite) and other mechanical properties.

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Appendix

Appendix I: Torque removal values after 2 weeks from implantation for all coated screws (N.cm).

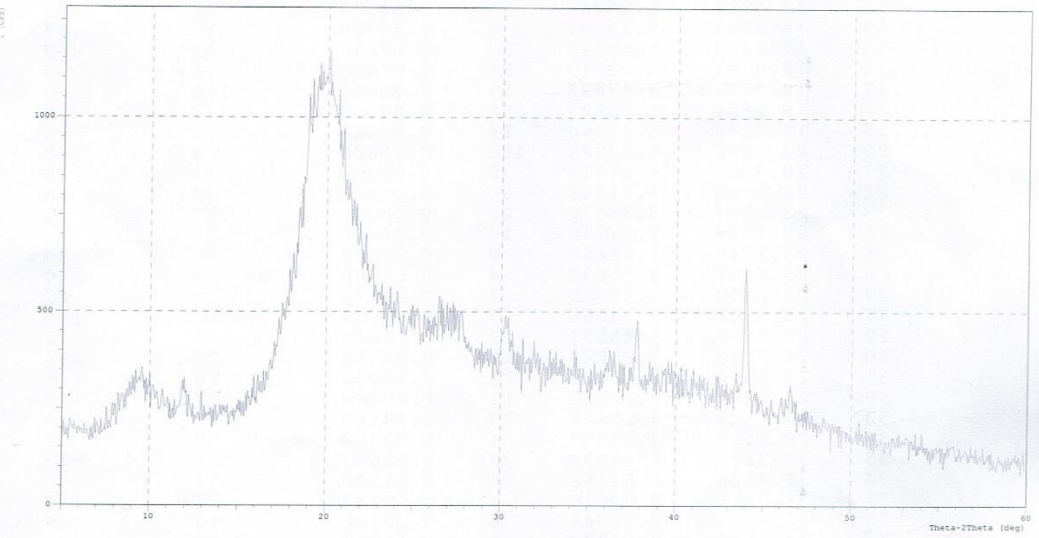
No.	Torque removal values for nano HA	Torque removal values for nano HA&chitosan mixture	Torque removal values for nano HA, chitosan &collagen mixture
1	12.00	17.65	21.18
2	14.12	21.18	17.65
3	10.59	14.12	17.65
4	17.65	21.18	28.24
5	14.12	21.18	24.71
6	14.12	17.65	28.24

Appendix II: Torque removal values after 6 weeks from implantation for all coated screws (N.cm).

No.	Torque removal values for nano HA	Torque removal values for nano HA&chitosan mixture	Torque removal values for nano HA, chitosan &collagen mixture
1	21.18	24.71	28.24
2	24.71	24.71	31.77
3	17.65	28.24	31.77
4	21.18	31.77	35.30
5	17.65	24.71	31.77
6	28.24	24.71	28.24

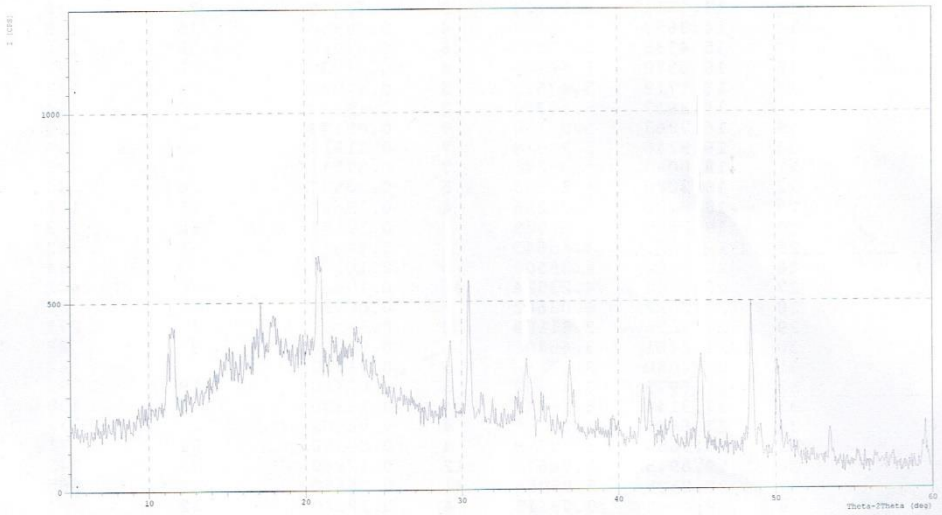
Appendix III: Chitosan XRD

```
                    ***  Analysis Condition  ***

      Group Name    : 12-15
      File Name     : 1240
      Sample        : 1240
      Comment       : 1240
      Date & Time   : 12-18-15 00:10:57

#Measure Condition
    XRAY
      Target      : Cu
      Wave        :  1.54060   (A)
      Voltage     : 40.0   (kV)
      Current     : 30.0   (mA)
    Slit
      Divergence : 1.00000   (deg)
      Scatter     : 1.00000   (deg)
      Receiving   : 0.15000   (mm)
    Measure
      Axis        : Theta-2Theta
      Scan Mode   : Continuous Scan
      Range       :    5.0000 -   60.0000 (deg)
      Step        :    0.0500   (deg)
      Speed       :    5.0000   (deg/min)
      Preset Time:  0.60   (sec)
```

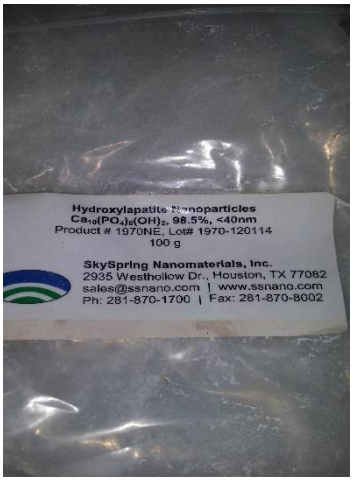


Appendix IV: collagen XRD

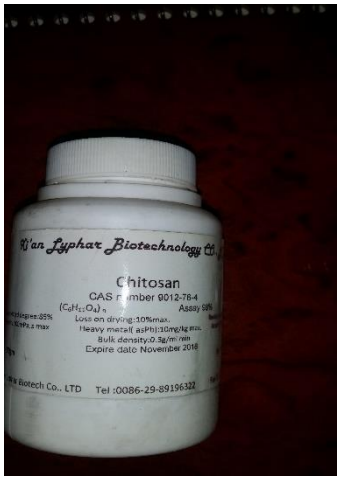
```
                    ***  Analysis Condition  ***

      Group Name    : 12-15
      File Name     : 1241
      Sample        : 1241
      Comment       : 1241
      Date & Time   : 12-17-15 23:56:49

#Measure Condition
    XRAY
      Target      : Cu
      Wave        :  1.54060   (A)
      Voltage     : 40.0   (kV)
      Current     : 30.0   (mA)
    Slit
      Divergence : 1.00000   (deg)
      Scatter     : 1.00000   (deg)
      Receiving   : 0.15000   (mm)
    Measure
      Axis        : Theta-2Theta
      Scan Mode   : Continuous Scan
      Range       :    5.0000 -   60.0000 (deg)
      Step        :    0.0500   (deg)
      Speed       :    5.0000   (deg/min)
      Preset Time:  0.60   (sec)
```



Appendix V :(A) Hydroxyapatite Nanoparticles ($Ca_{10}(PO_4)_6(OH)_2$), (B) Chitosan $(C_6H_{11}O_4)_n$ (C)Collagen.



(A)



(B)

(C)

الخلاصه

مقدمة: -في السنوات الأخيرة تطورت زراعة الأسنان و احتلت مكانة بارزة في مجال طب الأسنان، حيث توفر زراعة الأسنان الراحة و استعادة الوظيفة العادية تقريبا ، وتعتبر الخيار الأفضل لتعويض الأسنان المفقودة لجميع الأعمار من المرضى مقارنة مع التعويضات الاصطناعية القابلة للإزالة. واستخدمت تقنيات لتعديل الخصائص السطحية أو الطلاء لتحسين النجاح السريري لزراعة الأسنان.

الهدف من هذه الدراسة:- لتقييم تأثير مركب من الهيدروكسي ابتاتيت النانوي-االجيتوسان - الكولاجين عند طلائه على الزرعات المصنعه من التيتانيوم التجاري النقي على قوة الترابط العظام مع الغرسات عن طريق اختبار إزالة عزم الدوران ورد فعل الأنسجة عن طريق التحاليل النسيجية، و مقارنته مع طلاء الهيدروكسي ابتاتيت النانوي على التيتانيوم التجاري النقي.

الموادوطرق العمل: -قضبان من التيتانوم النقي التجاري تمت مكننتها الى (54 غرسه).كل غرسه صنعت بقطر 3 ملم طول 8 ملم (خمسة مليمات منها ملولب ثلاث مليمات ملساء)
تم تقسيم الغرسات إلى 3 مجموعات وفقا لأنواع من الطلاء المستخدمة: المجموعه الاولى تشمل (18 غرسه) طليت بواسطة طريقة التغطيس بمادة الهيدروكسي ابتاتيت النانوي, المجموعه الثانيه تضم (18 غرسه) طليت بالهيدروكسي ابيتايت النانوي مع الجيتوسان بطريقة التغطيس, اما المجموعه الثالثه(18غرسه) تم طلائها بطريقة التغطيس بمركب الهيدروكسي ابيتايت النانوي-الجيتوسان والكولاجين.
وقد تم تقييم خصائص السطح بعد طلاء من خلال فحص حيود الأشعه السينيه ,قياس سمك الطلاء, مجهر المسح الالكتروني, المجهر الضوئي الالكتروني ومطيافية الاشعه تحت الحمراء
عظم الساق لثمانية عشرمن الارانب النيوزلندية البيضاء اختيرت كمكان لوضع الغرسات. الساق اليمنى استقبلت غرستين الاولى مطليه بالهيدروكسي ابيتايت النانوي والثانيه مطليه بمركب الهيدروكسي ابيتايت النانوي والجيتوسان.اما الساق اليسرى فاستقبلت غرسه واحده مطليه بمركب الهيدروكسي ابيتايت النانوي-الجيتوسان والكولاجين

اجري فحص عزم التدوير لقياس قوة الترابط بين العظم والغرسة بعد اسبوعين و ستة أسابيع من فترة الشفاء, لكل فترة زمنية تم الفحص 18غرسة للعزم المطلوب لرفع الغرسة من العظم و9 غرسات للفحص النسيجي وبواقع 3غرسات لكل مجموعه.

النتائج:-اظهرت النتائج ان قيمة متوسط عزم الدوران للزرعات المطلية للفترة 2 و 6 اسابيع بمركب الهيدروكسي ابتاتيت النانوي ,الجيتوسان والكولاجين كانت اعلى من متوسط عزم الدوران لرفع الغرسات المطليه بالهيدروكسي ابياتيت النانوي وحده، ايضا كانت قيمة عزم الدوران لازالة الغرسات المطليه بمركب الهيدروكسي ابيتايت النانوي مع الجيتوسان اعلى من قيمة عزم الدوران للغرسات المطليه بالهيدروكسي ابيتايت وحده، بعد كل من فترات الشفاء وزيادة مع مرور الوقت لجميع الفئات المطليه.

كما تبين من خلال الفحص النسيجي ان تكوين العظم حول الغرسات كان اسرع للغرسات المطليه بمركب الهيدروكسي ابيتايت النانوي-الجيتوسان والكولاجين وسرعة نضوج العظم المتكون مقارنة مع باقي المجموعات.

الاستنتاج:- طلاء غرسات التيتانيوم التجاري النقي بمركب الهيدروكسي ابيتايت النانوي-الجيتوسان-الكولاجين كانت اكثر كفاءة من باقي المجموعات بزيادة قوة االارتباط بين العظم والغرسة والتي تبينت من خلال فحص قوة عزم التدوير ونسبة العظم الجديد المتكون في اسبوعين وستة اسابيع.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد-كلية طب الاسنان

تقييم تأثيرطلاء مركب من نانو الهيدروكسي ابيتايت والجيتوسان و الكولاجين على الغرسات المصنعة من التيتانيوم التجاري النقي (دراسة ميكانيكية ونسيجية)

رسالة
مقدمه الى مجلس كلية طب الاسنان في جامعة بغداد كجزء من متطلبات نيل درجة الماجستير في التعويضات الاصطناعية

من قبل
صابرين وليد ابراهيم
بكالوريوس طب وجراحة الفم والاسنان

اشراف
ا.د وداد عبدالهادي النقاش
بكالوريوس طب وجراحة الفم والاسنان
دبلوم تعويضات اصطناعية
ماجستير تعويضات اصطناعية

1438 هـ 2017 م