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**University of Baghdad**
**College of Dentistry**

# Studying the use of egg shell derived calcium carbonate as bone graft around nano calcium sulfate coated dental implant

A Thesis
Submitted to the council of the College of Dentistry at the University of Baghdad in Partial Fulfillment of the Requirements for the Degree of Master Science in Prosthodontics

By
**Dher Riyadh Kadhim**
B.D.S

Supervised by
Professor
**Dr. Thekra Ismael Hamad**
B.D.S., M.Sc., Ph.D. Prosthsodontics

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# Certification of the Supervisor

I certify that this thesis entitled "**Studying the use of egg shell derived calcium carbonate as bone graft around nano calcium sulfate coated dental implant**" was prepared by **Dher Riyadh Kadhim** under my supervision at the College of Dentistry/ University of Baghdad in partial fulfilment of the requirements for the degree of Master of Science in Prosthodontics.

Signature
Professor
**Dr. Thekra Ismael Hamad**
B.D.S., M.Sc., Ph.D. Prosthsodontics
(The supervisor)

# Linguistic certification

This is to certify that the thesis entitled **"Studying the use of egg shell derived calcium carbonate as bone graft around nano calcium sulfate coated dental implant"** was prepared under my linguistic supervision. Its language was amended to meet the style of the English language.

Signature:

**Prof. Dr. Abdulkarim Fadhil Jameel**

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Signature
Assistant Professor
**Dr. Abdalbasit A. Fatihallah**
B.D.S., M.Sc., Ph.D.
(Prosthodontics P.R.C. China)
(Chairman of Examination Committee)

Signature
Assistant Professor
**Dr. Amir Hussein Makki Khamal**
B.D.S., M.S.D.
(Prosthodontics)
(Member)

Signature
Assistant Professor
**Dr. Faiza M. Abdul-Ameer**
B.D.S., M.Sc.
(Prosthodontics)
(Member)

Approved by the council of the College of Dentistry/University of Baghdad.

Signature
Professor
**Dr. Hussain F. Al-Huwaizi**
B.D.S, M.Sc., Ph.D.
Dean of the College of Dentistry
University of Baghdad

# *Dedication*

*To the people I love the most:*

*To the memory of my father*

*To my mother*

*Who without her support this work would not have been accomplished*

*To my wife and my lovely son yasir*

*Source of love in my life*

*To my sisters and brothers*

*Source of support and encouragement*

*To every one who support me during this study…*

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# Abstract

**Background**

Dental implant insertion in regions with poor volume and quantity like posterior maxilla will lead to possibility implant failure in these regions. Calcium sulfate has been used as bone graft material due to its bone regeneration ability so it's used as coating material for Commercially pure titanium and to bind with egg shell powder as bone graft material around it to improve bone quality and quantity in bone defected regions.

**Aim of this study**

Dip coating evaluation for commercially pure titanium coated disc by nano calcium sulfate and to estimate and compare the effect of egg shell as a bone substitute on bone defect around commercially pure titanium coated implant with nano crystalline calcium sulfate mechanically by torque removal test, histologically and histomorphometrically.

**Material and method**

Coating properties of nano calcium sulfate film formed by dip coating technique on grade (II) Cp Ti discs by using different binders (Poly vinyal alcohol , Poly vinyal alcohol +Poly vinyal pyrrolidone) were estimated through: optical microscope, X-ray diffraction analysis, atomic Force Microscope, scanning electron microscope, energy-dispersive X-ray investigations and vickers microhardness measurements. Then evaluation of egg shell powder through X-ray diffraction analysis and fourier transfer infrared.

Regarding in vivo experiments, 80 screws designed- dental implants were used in the tibiae of twenty white New Zealand rabbits. twenty screws remain uncoated as control group and the remaining 60 screws coated by calcium sulfate nano particle, 20 screws coated used alone in the tibiae, for the other 20 coated screws , a gap made around them and filled by egg shell powder

as bone graft material, while for the last 20 coated screws , a gap made around them with out egg shell powder filling .

Finally, the screws were estimated biomechanically (through removal torque test) and biologically (through histological and hsitomorphmetrically examination) after 2 and 6 weeks healing periods .From each implants group, (8) implants were evaluated through removal torque test and (2) implants through histological observations for each healing periods.

**Results**

Dip coating technique for Cp Ti coated by calcium sulfate nanoparticle produce a coating film that improve surface microhardness value. Histologically, the screws that are coated by nano calcium sulfate used with egg shell powder as bone graft material around it showed to promote and enhance osseointegration level better than other groups, while histomorphometrically and mechanically after two and six weeks of implantations these screws showed significance difference as compared to other groups.

**Conclusions:**

**N**ano calcium sulfate can successfully work as coating material for titanium implant by dip coating method which increase surface microhardness and have uniform thickness of coating layer and significantly work with egg shell powder as bone graft material around it to increase the amount of new bone formation area in relation to other groups.

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# List of abbreviation

| *Abbreviation* | *Criteria* |
|---|---|
| AFM | Atomic Force Microscope |
| BW | Body Weight |
| BT | Bone Trabeculae |
| BIC | Bone-Implant Contact |
| CS | Calcium Sulfate |
| °C | Celsius |
| Co/Cr/Mo | Alloy Cobalt-Chromium-Molybdenum Alloy |
| Cp Ti | Commercially Pure Titanium |
| ES | Egg Shell |
| EDX | Energy-Dispersive X-ray |
| FR | Fibroreticular Tissue |
| FTIR | Fourier Transform Infrared Spectroscopy |
| G.P.T. | Glossary of Prosthodontic Terms |
| HV | Hardness Vickers |
| H.S | Haversian System |
| H and E | Hematoxylin and Eosin |
| HS | Highly Significant |
| HF | Hydrofluoric Acid |
| HCl | Hydrogen Chloride |
| HA | Hydroxyapatite |
| IACUCs | Institutional Animal Care and Use Committees |
| ICDD | International Centre for Diffraction Data |
| JCPDS | Joint Committee on Powder Diffraction Standards |
| Max. | Maximum |
| Min. | Minimum |
| nCs | Nano Calcium Sulfate |
| NBFA | New Bone Formation Area |
| N.cm | Newton. Centimeter |
| $HNO_3$ | Nitric Acid |
| NS | Non Significant |
| # | Number |
| OB | Osteoblasts |
| OC | Osteocytes |
| $P_2O_5$ | Phosphorous Pentoxide |

| | |
|---|---|
| PVA | Poly Vinyl Alcohol |
| PVP | Poly Vinyl Pyrrolidone |
| p-value | Probability Value |
| RTT | Removal Torque Test |
| RFA | Resonance Frequency Analysis |
| RL | Reversal Line |
| RTV | Reverse Torque Value |
| SEM | Scanning Electron Microscope |
| SPM | Scanning Probe Microscope |
| S | Significant |
| NaCl | Sodium Hypochlorite |
| SD | Standard Deviation |
| SE | Standard Errors |
| SPSS | Statistical Package for Social Sciences |
| XRD | X- Ray Diffraction |

# *Introduction*

# Introduction

Titanium dental implants are functional structures whose purpose is to restore edentulism **(Mich, 2008)**, in the past 10 years, many coating procedure and material were introduced to change the dental implant surface to get the best biological and biocompatibility properties, also to enhance and fasten the Osseointegration process which is regarded as the main feature for success of dental implant, precisely in patient complaing from defected bone healing **(Brett *et al.*, 2004; Palmquist *et al.*, 2010; Shukur 2014; Mandracci *et al.*, 2016)**.

With regard to the titanium and its alloys are broadly used in implant construction, however they may have a few negative aspect like minimum osseoinduction, highly corrosive due to low resistance to corrosion by product so coating the titanium and its alloy with bioceramic material like hydroxyl-appatite is one of the material that solve the issue (**Nie *et al.*, 2001**).

Many coating techniques have been emerged to enhance implant ability to engage healthy bone, the main principle of coating is to insert a thin bone substitute material that attach to the implant and bone while bone being supplemented **(Hench, 1993; Boyan *et al.*, 2001; Kasemo, 2002).**

Dip coating is regarded as one of the best procedure to get thin film of biocompatible ceramic layer on the titanium dental implant to enhance the osseointegration. This method are widely used, in respective to other procedures (spin coating), due to its highly controllable and widely relevant to big scale (sol, gel) preparation (**Aksakal *et al.*, 2008; Bosco *et al.*, 2012; Al-Hijazi *et al.*, 2013).**

Calcium sulfate is considered to be a synthetic ceramic group of bone substitute material due to osteoconductive ability. It is bio inert material when inserted inside the human body it will get resorbed and fibro vascular tissue appear in a period of weeks leading to neovascularization and bone formation

in the area, without significant inflammatory reaction **(Yashavanth, *et al.*, 2013).**

It has been proved that calcium sulfate may facilitate healing of human bone defect by surgical implantation as used by (**Peliter, 1961).**

Egg shell has been found to be a good bone substitute in maxillofacial branch, egg shell is regarded as biocompatible material, has the ability to bond with bony site and can be used (in peri-implant defect and interpostional graft) **(Dupoireux *et al.*, 2001; Dupoireux *et al.*, 1999; Dupoireux *et al.*, 1995).**

Calcium carbonate has been successfully recorded as biodegradable and osteoconductive. Also its resorbable in vivo, surface convertion to carbonate is not necessary in calcium carbonate like other bone graft material, in order to enhance bone formation **(Solmaz *et al.*, 2015; Guillemin *et al.*, 1987).**

# Aim of the study

The purposes of this study are to;

1. Evaluate the effect of dip coating of commercially pure titanium implant by calcium sulfate nanoparticles.
2. Evaluate the effect of egg shell as a bone substitute for bone defect around commercially pure titanium coated implant with nano crystalline calcium sulfate mechanically by torque removal test, histologically and histomorphometrically after 2-6 weeks healing period.

# *Chapter One*

# *Review of literature*

# Review of Literature

## 1.1 Bone

Bone is a connective tissue extremely unique composed from bone matrix, osteogenic cell (contain osteoblast, osteoclast and osteocyte) and vasculature. Inorganic and organic phase are the main component of bone matrix **(Junqueira *et al.*, 2005).**

### 1.1.1 Composition of bone

Inorganic constituent ranging from (60%-70%) of the dry bone weight is constructed epically from hydroxyapatite ($Ca_{10}$ $(PO_4)_6(OH_2)$ its plate like structure, solid and hardness properties are derived from this inorganic component **(Smith *et al.*, 2006; Wang *et al.*, 2010).**

Protein (collagen type I) and almost 5% matrix proteins non-collagenous type (osteocalcin, sialoprotin, ostioponetin, proteoglycans) which are the organic component of bone, several serum protein and growth factor available in the bone **(Nanci *et al.*, 2008; Wang *et al.*, 2010)**.

### 1.1.2 Classification of bone

On the basis of maturation, bone tissue has been grouped into: immature woven bone and lamellar bone, the immature woven bone is recognized by collagen fiber distribution irregularly although it contain smaller amount of mineral substance and higher proportion of osteocyte, immature bone eventually transferred to lamellar bone and considered as temporary bone **(Hadjidaskis, 2006; Wang *et al.*, 2010)**.

### 1.1.3 Bone cells

In normal bone usually three groups of cells are seen.

**A. Osteoblasts**

Osteoblasts cells contain only one nucleus, their function is a production of fresh bone by forming a new matrix of bone, osteoid and flowed by mineralization to form a new bone. Osteoblasts may act as a barrier that manages the ions transition into and out of bone also they make a sheet of cell precisely over the new bone **(Florencio *et al.*, 2015)**.

The hormones formation like prostaglandin which work on bone tissue is irresponsibility of osteoblast also osteoblasts supply an enzyme (alkaline phosphate) which is necessary for the mineralization process of bone **(Pratt, 2012; Saladin, 2012).**

**B. Osteocyte**

In bone production process many of osteoblasts bounded inside the bone matrix, they are secreted, whether mineralized or not and termed as osteocyte, which form almost (90%-95%) of total bone cell **(Nanci *et al.*, 2008).**

Osteocyte play a role in (matrix maintenance, homeostasis, production of bone and calcium), osteocytes work as sensation receptors to force and other stimuli. Thus they are concerned in both bone production and in modification of osteoblast formation **(Lian *et al.*, 2003; Muhonen, 2008; Wang *et al.*, 2010)**.

**C. Osteoclast**

Osteoclast play a significant role in bone remodeling processes because they control bone resorption which recognized by formation of enormous quantity of enzyme mainly lysosome like acid phosphate **(Nanci *et al.*, 2008).**

## 1.2 Osseointegration

Osseointegration is described as the direct attachment or connection of the host bone tissues to an exogenous and/or alloplastic materials without intervening or development of fibrous tissue at the bone-implant interface **(GPT , 2017).**

To get better osseointegrated titanium with high ability to success the implant must be:

1. Low trauma from the surgical technique during the insertion (no overheating of bone at implant site).
2. Initial stability should be confirmed when the implant placed.
3. The implant during the healing period of 3-6 months, should not be functionally loaded (**Block and Achong, 2004**).

Association of functional and esthetic result is believed to be the definition of successful osseointegration **(Albrektsoon *et al.*, 1986; Smith *and Zarb*, 1989).** Related to various factors, like appropriate bone quantity and quality, topography and roughness surface, non-occurrence of surgical complication, as bone contamination and overheating, occlusal overload and peri-implantitis **(Porter *et al.*, 2005; Panagakos *et al.*, 1996).** In regard to these causes, studies have been made to construct several biocompatible substances and a new coating technologies to increase osseointegration **(Rungsiyakull *et al.*, 2008; Mavrogenis *et al.*, 2009)**.

## 1.2.1 Mechanism of osseointegration

New bone formation on the implant surface is by sequent biological processes at the bone-implant interface (cellular and extracellular), these biological processes regulated by growth and differentiation agents, emitted at the operational site form activated blood cells ***(*Fini *et al.*, 2004).**

The bone healing process resemble the biological steps include the activation process, at least initial host response **(Rigo *et al.*, 2004; Soballe, *et al.*, 1993; Soballe, 1993)**.

The osseointegration of implant can take few months in human being and considered slow process, due to bone remodeling appear for reshaping or consolidation of bone-implant site, beside it produce a system for modification to self-cure and loads at the bone-implant site, to improve osseointegration of

implant learning all the steps that appear at (molecular and cellular grades) in the bone-implant interface is important **(Hofmann *et al.*, 1997 ; Dimitriou *et al.*, 2007)**.

The skeletal response after trauma has been well reported both mechanically and histologically due to its importance as it's a phenomena in molecular biology. After implantation the host response is altered by the presence of implant and its characteristic, the unintentional overheating injuries and the stability of fixation that contain death of osteocyte ranging from 100-500Um in to host bone **(Soballe *et al.*, 1993; Rigo *et al.*, 2004; Fini *et al.*, 2004).**

The main factors of peri-implant failure are the increased osteoclastic activity, the reduced quantity or / and effectiveness of osteogenic cell, the uncommon bone cell reproduction average and response to local and systemic stimuli, the imponderables among catabolic and anabolic local agents working on bone remodeling and generation, mechanical stress, and the impaired vascularization of the peri-implant tissue (**Marco *et al.*, 2005).**Vascularization considered as great value to the method of osseointegration. Osteogenic cell differentiation is depend upon tissue vascularity. Ossification likewise has direct relation with revascularization of tissue that begins to differentiate. As aging decrease angiogenesis, biomaterial osseointegration is also reduced. In the elderly, the failure risk is increased **(Marco *et al.*, 2005).**

The existence of bone marrow spaces or medullary including osteoclast, osteoblast, mesenchymal cell, lymphatic and blood vessels beside the implant surface is the confirmation sign of the turnover of peri-implant mature bone into osseointegrated implant. Through peri-implant bone remodeling process, new osteon perpendicular to the long axes of the implant and with their long axes parallel to the implant surface, also the new osteon circle around the implant. Osteoblast is responsible for osteoid production supposing that osteogenesis is underway. The reconstructed bone can expand about 1mm from the implant surface **(Franchi *et al.*, 2005; Chappard *et al.*, 1999).**

## 1.3 Dental implant

A dental implant can be described as prosthetic device of alloplastic material(s) that implanted 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 dental prosthesis; a substance that is placed into and/or on the jaw bone to support a fixed or removable dental prosthesis **(GPT, 2017).**

### 1.3.1 Classification of materials used as dental implants

Branemark is first one who introduce oral implant for the replacement of missing teeth in 1960 **(Branemark *et al.*, 1997)**. The chemical and physical properties of implant material are well documented and reported elements that effect the prognosis of implant therapy and clinical result. Which include surface composition, the microstructure of the implant, as well as design factor **(Smith, 1993).** The implant material called ideal when it have the following features: strength, biocompatible, corrosion resistant, appropriate toughness, fractures and wear resistance **(Smith, 1993; Parr *et al.*, 1985).** The physical properties should be compatible with principle design of the implant. Materials that are used in implant construction can be grouped on the basis of the biological response they elicit when implanted or chemical composition **(Sykaras *et al.*, 2000).** From chemical vision, dental implant made from ceramic, metal or polymer table

**Table 1.1: Chemical classification of implant material (Berner *et al.*, 2009).**

| Implant Material | Common Name or Abbreviation |
|---|---|
| I. Metals | |
| Titanium | CpTi |
| Titanium Alloys | Ti-6A1-4V extra low interstitial (ELI) |
| Ti-6A1-4V | |
| Ti-6Al-7Nb | |
| Ti-5Al-2.5Fe | |
| Ti-15 Zr-4Nb-2Ta-0.2Pd | |
| Ti-29Nb-13Ta-4.6Zr | |
| Roxolid (83%–87%Ti-13%–17%Zr) | |

| | |
|---|---|
| Stainless Steel | SS, 316 LSS |
| Cobalt Chromium Alloy | Vitallium, Co-Cr-Mo |
| Gold Alloys | Au Alloys |
| Tantalum | Ta |
| II. Ceramics | |
| Alumina | $Al_2O_3$, polycrystalline alumina or single-crystal sapphire |
| Hydroxyapatite | HA, $Ca_{10}(PO_4)_{10}$, $(OH)_2$ |
| Beta-Tricalcium phosphate | β-TCP, $Ca_3(PO_4)_2$ |
| Carbon | C |
| vitreous, | |
| Low-temperature isotropic (LTI), | |
| Ultra-low-temperature isotropic (ULTI) | |
| Carbon-Silicon | C-Si |
| Bioglass | $SiO_2/CaO/Na_2O/P_2O_5$ |
| Zirconia | ZrO2 |
| Zirconia-toughened alumina | ZTA |
| III. Polymers | |
| Polymethylmethacrylate | PMMA |
| Polytetrafluoroethylene | PTFE |
| Polyethylene | PE |
| Polysulfone | PSF |
| Polyurethane | PU |
| Polyether ether ketone | PEEK |

### 1.3.2 Commercially pure titanium (CP Ti) and its alloys

There are six distinct groups of titanium used as implant biomaterials, Approved by the American Society for Testing and Materials (ASTM). From these six materials, there are two titanium (Ti) alloys and four stages of commercially pure titanium (Cp Ti). The physical and mechanical features of Cp Ti are dissimilar and are linked mainly to the residuals of oxygen in the metal.

The two alloys are Ti-6Al-4V-ELI (extra low interstitial alloys) and Ti-6Al-4V. Cp Ti materials are termed as pure (Grade I, Grade II, Grade III and Grade IV)

titanium. Cp Ti is described as unalloyed titanium and generally include residual elements of (nitrogen, oxygen, iron and carbon). These residual chemical agents enhance mechanical properties of pure titanium which founded in enormous quantity in all grades of titanium **(McCracke *et al.*, 1999)**.

Commercially pure titanium (Cp Ti) and extra low interstitial Ti-6Al-4V (ELI) are the most two usual titanium implant biomaterials. These two types of titanium are categorized as biologically inert biomaterials. Since they keep stability after implantation inside human bodies. The defense mechanism is capable of diagnosing titanium as foreign bodies and attempts to separate titanium by covering it in fibrous tissues. But, Titanium do not produce negative feedback and are handled well by the human tissues. Allergic reaction not induced by these material as it induced by stainless steels, especially nickel hypersensitivity in neighboring tissues (**Oldani and Dominguez, 2012).**

**1.3.2.1 Physical properties of CP Ti**

Titanium is a low corrosion metal with a high strength light weight. Titanium is a chemical elements and its color silver gray of group IVb of the periodic table **(Gonzalez and Mirza-Rosca, 1999).**

Cp Ti is found in four grade because oxygen and iron differences in contents and the physical properties of titanium. The oxygen increase strength and ductility of Cp Ti however iron is add to enhance the residual agents (carbon, hydrogen, nitrogen), count for stability improvement or enhancement of the physicochemical and mechanical properties **(Park *et al.*, 2000; Saini *et al.*, 2015).**

The modulus of elasticity of titanium almost similar to bone tissue, except it has minimum wear and shear strength and abrasion resistance, as well it is stronger than steel but lighter **(Freese *et al.*, 2001; Liu *et al.*, 2004).**

**Table 1.2: Summary of some physical properties of 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, $\alpha$, at 20 8C ($K^{-1}$) | 8.4 ×$10^{-6}$ |
| Melting temperature (C) | 1668 |
| Thermal conductivity (W/(m K)) | 19.2 |
| Boiling temperature (estimated) (C) | 3260 |
| Transformation temperature (C) | 882.5 |
| Electrical resistivity<br>High purity (mV cm) 42<br>Commercial purity (mV cm) | <br>42<br>55 |
| Modulus of elasticity, $\alpha$, (GPa) | 105 |
| Yield strength, $\alpha$, (MPa) | 692 |
| Ultimate strength, $\alpha$ , (MPa) | 785 |

## 1.5 Bone-implant interface

### 1.4.1 Definition

Interface is described as a flat forming the usual boundary between two section of space or matter **(Rigsby *et al.*, 1998)**. It may consist of region or zone of interaction between two particles or may represent discrete border between two materials like the interface that appear between implant and bone. The interface of implant-to-tissue is highly dynamic region of interaction, which it change in character completely as it changes from its origin (implant insertion into planned bone site) to its healed state (maturity) **(Rigsby *et al.*, 1998)**.

The fibro-osseous interface developed when there is a micromotion among the bone and implant at implant placement time. The curing period of the interface is just the state of its dynamic nature **(Rigsby *et al.*, 1998).**

The surgical method is done on the patient to place implant (foreign material), also the body is approached to recuperate the injury **(Rigsby *et al.*, 1998).**

A detailed understanding of the response behavior of highly differentiated hard and soft tissue to surgical preparation of the host site and construction of the prosthesis, also the long-term tissue adaptation to functional demand on the anchorage unit are the essential principles for building true and durable tissue integration of a non-biologic prosthesis with low risk of negative reaction **(Rigsby *et al.*, 1998).**

## 1.4.2 Importance of bone-implant interface

The fundamental drivers for various implant design is to enhance bone-implant engagement. Osseointegration associate with overlapping of the bone with the feature on the implant on the implant surface at the micro and macro-level, as well as chemical bonding of the tissue to the implant. Linkage strength and decreased healing time has been improved by morphological and chemical modification of the implant surface. Hydroxyapatite which is a bioactive ceramic has been used as coating material on the implant surface. Which enhance bone to implant stabilization through chemical bond among the surrounding tissue and implant. Surface morphology alteration in macro-level by undercuts, design of thread, layer of wire and in micro-level by rising surface roughness **(Faegh *et al.*, 2011).**

Chemistry and shape of the bone-implant interface affect the transportation of stress in the bone for different implant designs **(Bidez *et al.*, 1988; Hipp *et al.*, 1985**). **Siegele and Soltesz (1989)** planned to use frictional contact to model different bone –to-implant connection type. Their result showed that high stresses level appear in the apical area of the implant if typical osseointegration is

confirmed, as well as the high stress area appear in the alveolar crest for frictional sliding contact condition **(Bidez *et al.*, 1998; Hipp, *et al.*, 1985)**.

There are many factors affecting bone implant –interface which include implant material biocompatibility, implant surface and the design, the condition of the host, the surgical technique of implant placement and situation of loading utilized afterward **(Albrektson *and* Albrektson, 1987; Braceras *et al.*, 2002; Mavrogenis *et al.*, 2009)**.

### 1.4.3 Dental implant stability

Implant stability is a measure of clinical immobility and considered as indirect sign of osseointegration **(Atsumi *et al.*, 2007)**.

Primary stability usually appear with cortical bone from mechanical attachment. Secondary stability gives biological stability by bone remodeling and regeneration (**Brunski *et al.*, 1992; Sennerby *et al.*, 1998**). Primary stability is concerned by bone amount and nature, surgical method and implant geometry (width, length, surface characteristics). Secondary stability is affected by primary stability **(Cochran *et al.*, 1998**).

Four weeks after implantation the secondary stability has shown to begin to increase **(Davies, 1998).**

#### 1.4.3.1 Methods of measurement of dental implant stability

The destructive or nondestructive are different methods used to assess implant stability **(Meredith, 1998)**.

#### 1.4.3.2 Destructive methods

**A -Histomorphometric analysis**

This is obtained through measuring the amount of pre-implant bone and the bone to implant contact (BIC) from a non vital sample of the pre-implant bone and implant. Correct calculation is a superiority, but because the destructive and invasive method, it is not applicable for longstanding reports. It is applied in the experiments, nonclinical studies. It is helped at before, during and after surgical time **(Nkenke *et al.*, 2004).**

**B. Push-out / pull-out test**

Inspect the curing abilities at the interface of bone to implant, it record interfacial shear strength by load administration parallel to the interface (bone-implant). In this test a cylindrical implant is inserted intramedullary or transcortically inside bone and detached by applying a load to the interface parallelely (**Brunski *et al.*, 2000).** The failure load or highest load capabilities is explained as the highest force on displacement scheme of force, interfacial stiffness is recorded when tangent scheme at the linear region of the load displacement curve before breakpoint. The main disadvantage of this test is only used for non-threaded cylindrical type implant, while the majority of clinically used implants are of threaded design **(Brunski *et al.*, 2000; Chang *et al.*, 2010).**

**C. Tensional test**

Previously the tensional test was calculated by removing the implant fixture from the bone. Later Branemark modification was done by using lateral force to implant plate. Yet, the scientist found the test could not be translated easily to any region of independent mechanical properties **(Meenakshi *et al.*, 2013).**

**D. Removal torque test**

Removal torque test was done to estimate the stability of implant in the bone **(Suh *et al.*, 2007).** Removal torque test regarded as a useful tool for analysis the screw-shaped implant. So this test was included in many studies **(Cheul-Goo *et al.*, 2015; Lee *et al.*, 2016; Park, *et al.*, 2016).**

Torque is the movement or twisting applied by load at a span on body identical with load multiplied by the horizontal span between the center of rotation and line of action at which its applied **(Yousef *et al.*, 2005).**

The anchorage between bone and implant is calculated by removal torque test, the strength of osseointegration is increase, as more torque needed to remove the implant. So, when bone-implant contact is destructed the (RTT) can measure the critical torque threshold **(Atsumi *et al.*, 2007**; **Elias *et al.*, 2008).**

Incidence of early loading failure during the first year of loading was reduced by using reverse torque testing **(Jividen *et al.*, 2000).** Reverse torque value effected by many factor when unscrewing of titanium implant: such as geometrical implant surface variation, interface tissue structure and the nature of neighboring bone **(Sennerby *et al.*, 1992).**

A study in four handered and four implants osseointegrated clinically revealed that the removal torque value ordered from 45N.cm to 48N.cm. Since the (RTV) known to be a reliable source of osseointegration verification but it is damaging test and applied chiefly in experimental animal studies **(Sullivan *et al.*, 1996; Atsumi *et al.*, 2007)**.

**1.4.3.3 Nondestructive methods**

**A. Percussion test**

One of the simplest processes that can be applied to estimate the degree of osseointegration, which depend on impact-response theory and vibrational acoustic science. Evaluation done clinically by percussion with a metallic instrument to investigate the heard sound, crystal (ringing sound) means that the osseointegration is successful, while no osseointegration remarked by dull sound. The percussion test depend greatly on experience level and skill of the clinician, so it can't be applied as standardized test for experimental method **( Atsumi *et al.*, 2007; Bayarchimeg *et al.*, 2013).**

**B. Insertion torque measurement**

Bone amount in several regions of the jaw at same time of implant insertion has been measured by insertion torque value. Insertion torque can be applied only as separate tool to measure the stability, although it regarded as a changeable, influencing stability. In various light source, insertion torque considered as mechanical scale mostly influenced through implant design, bone nature and surgical method at the implant site **(Irinakis *et al.*, 2009; O'Sullivan, *et al.*, 2004**).Yet, it can't measure the secondary stability by remodel around the implant and new bone production. Thus it can't record longitudinal information to

measure implant stableness change later on implantation. Moreover, if insertion torque increased it mean that primary stability is increased, although the insertion torque highest value reached when implant neck produce a pressure on alveolar bone **(Bayarchimeg *et al.*, 2013).**

**C. Periotest**

Periotest is a machine use to overwhelm the problem that are related to other destructive method of implant stability measurement **(Ito *et al.*, 2008)**. By using an electronically controlled tapping metallic rod in a handpiece and electromagnetically driven. A small accelerometer included in the head can measure response to striking **(Atsumi *et al.*, 2007).**

The main limitation of periotest clinically are: such as failure to locate the mobility in mesial and distal direction, angle of the rod cannot be detected easily and measured, implant bone interface small differences cannot be located along with the tight zone through the parameter of periotest device, thus it has minimum critical information concerning the stability of dental implant **(Ito *et al.*, 2008; park *et al.*, 2011)**.

**D. Resonance frequency analysis (RFA)**

It was firstly proposed by **Meredith in 1998.** It is a non-destructive procedure that calculate bone density and implant stability at different time marks using a principle of structural analysis and vibration. RFA employ a transducer that has resemble the (L) letter and it's fixed by screw with implant or abutment. This transducer has two elements of ceramic: one element is pulsated by a frequency ranged from (5-15) Hz, while the second has a function of a receptor **(Aparicio *et al.*, 2006).**

The resonance frequency analysis can be applied for observation of difference in implant stability (long and short term observation) **(Huwiler *et al.*, 2007; Tozum *et al.*, 2007; Capek *et al.*, 2009)**.

**Huang *et al*. in 2005** proved this analysis is an efficient when used for diagnosis reasons to check implant stability in clinical and nonclinical research.

**E. Radiological tests**

Ridographical investigation is non-destructive method can be done at any level of healing. It has been documented in the first year of loading in a stable implant the expected crestal bone loss is 1.5mm radiographical, with 0.1mm of subsequent annual bone loss **(Adell *et al.*, 1981; Albrektsoon *et al.*, 1986; Smith *et al.*, 1989).** Conventional radiograph has many precaution in making an accurate, independent assessment of implant stability **(Atsumi *et al.*, 2007).**

Radiographical view like the bitewing view can be applied to estimate the crestal bone level which regarded as radiographical indicator for implant success **(Attard *et al.*, 2005).** Conventional panoramic or periapical views don't produce information on bone loss at facial bone level precedes mesiodistal bone loss **(Micsh, 2005).**

Neither bone density nor quality can be measured by this method, Also bone mineral can't radiologically diagnosed till 40% of demineralization had appeared (**Goodson *et al.*, 1984).**

## 1.5 Titanium implant surface chemistry:

The chemical stability and structural integrity of titanium oxide passive films are related to titanium biocompatibility, also the resistance of corrosion is dependence on passive film, titanium stability and electromechanical properties is heavily related to the main component structure and thickness of oxide film. The passive film will appear in (9-10) sec **(Song *et al.*, 2004 ; Saini *et al.*, 2015).**

**Sul *et al*** investigated a series of studies the effect of surface chemistry in vivo, who invented alleged bioactive implant surface and novel-electro chemical method **(Sul *et al.*, 2006; Sul *et al.*, 2006).** Incorporation of sulfur to the implant surface **(Sul *et al.*, 2002)** incorporation of calcium, phosphorous and magnesium **(Sul *et al.*, 2007).**

Bone response significantly improved with oxidized implant compared with dual-etched implant and machine-turned implant. The previous studies, suggest that mg-incorporated oxidized implant contain biochemical bonding connect implant to bone, producing a strong and fast integration, precisely at healing time less than 6 week, this bonding will support bone connection in pits, pore and implant surface irregularities, producing fast implant stability **(Sul *et al.*, 2007).**

## 1.6 Properties of an implant biomaterial

Bio materials are those materials that are accepted by living tissues and can be used for tissue replacements. On a macroscopic level these devices are used to fix or replace a bone and to support its healing process.

With the worldwide increase in the average age of population there is a subsequent increase in the number of surgical procedures which has in-turn urged researchers to improve and optimize bio materials **(Mich, 1999; Mudduganadhar, 2011; Daniel Glad Stephen *et al, 2017*).**

**A-Modulus of elasticity**

For decreasing movement in the region of bone-implant interface and to confirm equal stress transportation to the implant, modulus elasticity of implant material must be comparable to (18 Gpa) **.**

**B-Tensile, compressive and shear strength**

The material of implant must have maximum compressive strength and high tensile to improve functional stability and prevent fracture. Interfacial shear strength is recorded to increase when enhanced force transfer from the implant to bone and lower force in the implant.

**C-Yield strength and fatigue strength**

Preventing fragile break in status of cyclic loading by achieving high fatigue strength and yield strength in the implant material.

**D-Ductility**

Implant ductility is important for shaping and contouring of an implant, ADA (American Dental Association) stated that minimum ductility for dental implant is 8%**.**

**E-Hardness and Toughness**

Increase of wear ratio in implant material when the hardness is decreased and implant fracture is related to decrease in toughness value.

## 1.7 Surface properties

The future success of dental implants depends on the reaction of the dental implant surface with living human tissue. Therefore, the surface properties and the reaction of the dental implant with the tissue is critical in determine the success of implantation. Biologically, the host bone response is influenced by the titanium implant surface. This surface is merely part of the dental implant that contacts the host bone, and differs from the main body of the dental implant in many aspects **(Bauer *et al.*, 2013).**

**A-Surface tension and surface energy**

Implant wettability described by cleanliness of implant surface and wetting fluid (blood). Improved adhesion of osteoblast over the implant surface. Surface energy has a direct effect on protein adsorption **(Misch *et al.*, 1999)**

## B-Surface roughness

Surface roughness manipulation in implant effect the tissue and cell response by raising implant surface area beside bone and therefore enhancing attachment of cell to the bone. Surfaces of implant grouped according to the surface properties, like texture, roughness and orientation of irregularities **(Wennerberg and Albrektsson, 2010, Chaturvedi, 2009).**

## 1.8 Implant surface modifications

Surface manipulation is usually done to stabilize the preferable feature of implant material.

Survival rate of titanium implant in long term are excellent, even though implant collapse will occur in small number of patient, first reason because inadequate osseointegration approximately 1-2% of patient with in early months, second reasons appear in few years after good osseointegration almost 5% of the patient and frequently result from peri-implantitis (**Chrcanovic *et al.*, 2014; Smeet *et al.*, 2014).**

Also there are many reports on patient with systemic disease like (diabetes mellitus, osteoporosis, etc) have impaired osseointegration result, these situations are challenging in oral implantology and demanding surface adjustment bioactivity to fasten osseointegration process after implant surgery **(G´omez-de Diego, 2014**).

The principle purpose of modification in the surface of implant is to speed up the early osseointegration and provide a stable bone to implant contact with no evidence of extraordinary marginal bone destruction **(Smeets *et al.*, 2016).**

Appositional bone growth is the way by which smooth surface integrate, bone follow implant surface directly in microrough surface to accommodate (contact osteogensis) **(Junker *et al.*, 2009).**

In the smooth surface of implant show close adherence to the fibroblast and epithelial cell and the rough surface is more related to the synthesis of collagen and proliferation of osteoblast **(Cochran *et al.*, 1998; Albrektsson *et al.*, 2004).**

The disadvantages of smooth dental implant are: blood colt retraction will cause a space between implant and bone, thus cells are unable to reach implant surface. So, faster integration rate has been found in moderately rough surface with increased bone contact as compared to the smooth surface **(Ivanoff *et al.*, 2001; Zechner *et al.*, 2003; Sennerby *et al.*, 2008).**

Several studies used dental implant for medical trial reveal that rough surface bond better with bone than smooth surface **(Cochran, 1999).**

Poor quality of the bone is the primary indication for using rough surface of implant. Surface roughness of implant can be grouped into three groups regarding the dimension of surface **(Smeets, 2016)**:

1-Macro roughness feature: it's defined by the visibility of its geometry like threads and designs of thread which have almost the parameter from millimeter to ten micron, satisfactory hole drilling of the implant combine with proper macro roughness of the surface is essential for clinical success of oral implantology **(Smeets *et al.*, 2016)**.

2-Micro-roughness feature: on the micro-roughness order. The best engagement of bone to the fixture surface can appear when micro-roughness on scale (1-100um) **(Brett *et al.*, 2004; Smeets *et al.*, 2016)**.

3-Nano-sized roughness: have the dimension of (1-100) nm have an important function in cell to implant reaction at the protein and cellular level (osteoblast action and protein adsorption), this might improve rate of osseointegration **(Brett *et al.*, 2004; Smeets *et al.*, 2016)**.

Surface modifications contain topographical and chemical modification, the topographical like roughness, while chemical like coating **(Ehrenfest *et al.*, 2010; Mandracci *et al.*, 2016)**.

## 1.8.1 Types of implant surface modifications

Various techniques have been emerged and applied to modify implant surface geometry, these techniques include:

**A. Machined surfaces**

Transformation of titanium into the cylinder or screw is by feeding a long rod of titanium into a machine and that will create a machine surface which characterized by 10 micron (groove or ridges) deep in the surface **(Koshy and Philp, 2015).**

The term machined surface in implantology is commonly used as definition for a milled, turned or occasionally polished surface **(Wennerberg *et al.*, 2000).** The advantages of pit , groove and line that used usually in the machined surface is facilitating the adherence of osteoblast and speed up the rate of formation and connection of bone tissue to machined surface these feature have a great influence on osseointegration **(Piller, 1998 ; Albrektsson *et al.*, 2004; Muddugangadhar *et al.*, 2011)**.

**B. Chemically etched surface**

Strong acid usually used as etching material (HCl, $H_2SO_4$, $HNO_3$ and HF) to obtaining roughening titanium implant. The effect of acid etching are maximizing the surface are by formation micro pits (0.5-2mm) in diameter on titanium surface also it has been reported that acid etching will increase osseointegration (**Lausmaa, 2001; Mandracci *et al.*, 2016)**.

**C. Anodic oxidation**

Anodization methods changing in crystal nature of titanium oxide layer and the the micro-framework (**Sul *et al.*, 2002**). Anodization of the surface will cause in a great support in bone reaction by higher rate for bio-mechanical and histomorphetric test in regard to machined surface, a high success ratio was reported in an anodized titanium implant in relation with turned titanium surface of exact shape (**Junger *et al.*, 2005).**

**D. Porous sintered surfaces**

Polymeric sintering of titanium and metal alloy in to a machined titanium surface using high temperature and controlled atmospheric pressure to create a surface that is uniform in porosity which greatly increase surface area and this surface identified by induction of bone ingrowth fastly and the osseointegration is more improved **(Piller, 1998; Al-Ma'adhidi, 2002; Wang *et al.*, 2016)**.

**E. Thermal spraying processes**

Application of non-metallic or metallic coating is done by thermal spray, which is common expression for coating techniques groups that can be classified into three

types: electric arc spray, plasma arc spray and flame spray **(Knight and Smith, 1998).**

Is used to coat material over each other by heat and velocity, employing powder, molten metal, wire as feedstick, ceramic or molten alloy are the material of choice for substrate material **(Herman and Sampath, 1993; Levingstone, 2008)**.

**F. Laser etching technique**

The laser procedure result in surface with adequate roughness and better osseointegration and minimum contamination (**Gaggl *et al.*, 2000**), A study done in rabbit tibia had showed that increased of removal torque, with implant treated with laser etching technique in comparison to turned surfaces ( **Cho and Jung, 2003**).

**G. Ion implantation method**

Is a process applied to spray ions into surface. Several ions can be planted with planned concentrations to variable depths of the substance Implantation of nitrogen ion is the most usual ion implanted by this technique. **(Lee *et al.*, 2001; Rautray *et al.*, 2010).**

**H. Dental implants coating**

Surface manipulation, by using different procedure like coating technique able to raise the contact between the surface of dental implant and bone this will result in maximum biomechanical stabilization at early stages of implantation in relation to machined dental implant **(Coelho *et al.*, 2009)**.

A sufficient coating surface should have a tendency to do the followings: cell differentiation, improve cell attachment and bone apposition: limit the rate of dissolution in the body fluid, function in therapeutic way and bone fixation **(Junker *et al.*, 2009).**

After coating is done on Ti implant, the coating material is located between bone surface and the Ti implant. All forces can be tolerated by coating of implant when

it's subjected to load during transferring all the load imposed on the implant **(Oshida *et al.*, 2010; Tomisa, *et al.*, 2011).**

## 1.9 Implant coating techniques

Improvement in the surface characteristic require not only a new coating material but also demand technologies and techniques used to deposit these material on the implant surface have observed advancement in last decades, so for coating methods various material have been used along with numerous deposition procedure have been inspected: thermal spraying, sol-gel deposition, electrophoretic deposition, laser deposition and biomimetic deposition **(Barrère *et al.*, 2008; Scholz *et al.*, 2011; Smeets *et al.*, 2016).**

There are many advantages and disadvantages of the usual coating technique as mentioned by **Bosc *et al.*, in 2012** in the following table (1.3).

**Table 1.3: Some of advantages and disadvantages of commonly used techniques of coating for implants (Bosco *et al.*, 2012).**

| Technique | Advantage | Disadvantage |
|---|---|---|
| **Plasma spraying** | maximum deposition rate | Non- uniform coating crystalinty |
| **RF magnetron sputtering** | Uniform and dense coating :strong adhesion | Time consuming :low deposition rate |
| **Pulsed laser deposition** | Controlling the overall coating morphology and chemistry | Line of sight technique |
| **Ion beam dynamic mixing deposition** | High adverse strength | Require high temperature for sintering and line of sight technique |
| **Ion beam assisted deposition** | Increased tensile bond strength | Line of sight technique |
| **Biometric deposition** | Coating of complex geometric :co-deposition of biomolecule | Time consuming: require controlled PH |
| **Sol –gel deposition** | Coating of complex geometric: low processing temperature | Low processing temperature require controlled atomosphere processing : expensive raw material |
| **Electrophoretic deposition** | Complex geometries: high deposition rate co- deposition of biomolecule | Crack free coating is difficult to produce with low adhesiveness |
| **Electrospray deposition** | Control over coating composition and morphology | Low mechanical strength line or sight technique |

## 1.10 Nanotechnology and implant surface nanophase coatings

Nanotechnolgies has been defined the procedure that produce structure and material with designed feature in 1-100 nm in size range (**Whitesides *et al.*, 1991; Niemeyer, 2002).**

Nanotechnology has necessary part in engineering field of the surface: like minimum coating thickness (less than 100nm) or formation of nanostructured coating by using nano-crystal or nano particle also with minimum dimension less than 100 nm. The expanding scope of topographical modification from the micro-scale to nano-scale is the aim of nanotechnology which affects in the biomolecule's, cell and ion at the nano-sccale **(Christenson *et al.*, 2007; Mendonça *et al.*, 2008).**

Controlling and understanding nano level interfacial reactions is the crucial to produce new surfaces which will remove refusal and improve integration and adhesion to the neighboring tissue.

Nano-biomaterial provide surface area relatively higher than the conventional one, so the nano ranged surface enhance the wettability of surface to blood by surface energy also the adherence and expanding fibrin and fiber and protein matrix on the surface of implant this regarded the reason its highly effective physically during primary stages of healing –The attachment of cell is largely improved and enhanced tissue curing on the implant-interphase **(Mendonça *et al.*, 2008)**.

Generally, three causes explain the bond adhesion increasment and lifecycle of implants that have nano-surfaces **(Keshmiri *et al.*, 2003; Palin *et al.*, 2005)**.

- Nano surface implant can produce effective action of osteoblast by limiting natural bond surfaces.
- Titanium oxide group on the nano surfaces implants are in great thickness, thus enhancement in apatite nucleation.

- Nano surfaces implants have more surface area can improve proteins adsorption that are required for the adherence of osteoblast.

Nanoparticle can be defined as any ultra-fine molecule with range (1-100)nm in dimension composed essentially by any biocompatible substance, as compared to its similar bulk material **(Hallaj-Nezhadia *et al., 2010;* Lotfipour, *et al.,* 2014; Dizaj *et al.,*2015).**

Nanoparticles have been recorded to be used to strength bone fusion of dental implant (**Samiei *et al.,* 2016).**

Nanoparticle can be used for implant coating purpose to increase dental implant success and improve soft tissue integration. Dental implant coated by nanomaterial with osteoconductive ability could result in a chemical bond with bone to gain accepted biological stabilization **(Chidambaranathan, *et al.,* 2016).** Regeneration potential and bone regeneration of nanomaterial, as well can produce a positive circumstances for bone production. Bio-inert nanomaterial coating (aluminum and zirconium) on the implant surface may alter the properties physically and improve osteogenic ability of implant (**Chidambaranathan, *et al.,* 2016).**

## 1.11 Calcium sulfate coatings

Calcium sulfate hemihydrate used for many years in medicine and dentistry as bone graft material (**Peltier, 1959**).

Calcium sulfate is considered as bone graft binder material for more than 110 years **(Peltier, 1961).**

Calcium sulfate considered as bone substitute material because its degradation properties releasing calcium ion in vivo situation forming calcium phosphate and dropping of PH temporarily, as a consequence to this demineralization in the surface of bone occur leading to growth factor release like bone morphogenetic and transforming growth factor which enhance bone growth in the defect (**Walsh *et al.,* 2003**).

Experimental studies showed that nano-sized ceramic material can be used as bone substitute material because their excellent osteogenic properties **(Webster *et al.*, 2000; Arts, *et al.*, 2006).**

Nano calcium sulfate has emerged with much smaller size than conventional one and have the ability of sustained release plus slower rate of resorption in comparison to medical grade calcium sulfate (**Park, *et al.*, 2011**).

Calcium sulfate is alloplastic material and considered biodegradable, biocompatible, osteogenesis stimulator with rapid resorption rate (**Thomas *et al.*, 2001; Kelly *et al.*, 2001; Gitelis, 2001).**

Its stimulate the formation of blood vessel and no inflammatory reaction has been recorded **(Paolantonio *et al.*, 2008; Strocchi *et al.*, 2002),** when calcium sulfate work as a binder in combination with other graft material (particle-based type) leading to raise bone production, better handling characteristic, improve molecule graft containment, this will lead to more bone production to the defected site as compared to allograft material alone **(Al ruhaimi, 2000).**

Calcium sulfate has the tendency to produce space for the osteoprogenitor cell to heal and proliferate by long lasting action, it is regarded as calcium provider for mineralization of the newly formed bone which is essential for healing **(Beeson, 1981).**

Calcium sulfate has biological properties that induce bone formation (osteoconductive) without using other bone graft with calcium sulfate, also it is used as delivery transporter in drugs and growth factor **(Ricci *et al.*, 2008),** calcium sulfate reported to work in endodontic defect repair like apicectomies **(Conner, 1996; Kim *et al.*,1997)**, several reports indicate the use of calcium sulfate for repairing orthopedic defect (**Hadjipavlou *et al.*,2000; Gitelis, *et al.*, 2001;Kelly *et al.*, 2001; Mirzayan *et al.*, 2001; Kelly and Wilkins,2004).**

# 1.12 Sol-Gel deposition

Is a procedure of formation particle of small size in material chemistry especially used for production of metal oxide **(Kumar *et al.*, 2015).** Also described as a group of experimental steps as follow **(Ganguli, 1993):**

1-Solution preparation by generation of ultra-fine in liquid vehicle in situ.

2-Develop the solution with suitable properties to get desirable properties.

3-The sol after that submitted to, spinning, casting, coating, drawing, spraying, dipping, emulsification , etc. for gaining the needed gel shape coating by sol gel transformation.

4-The final step is heating and drying to have the desirable properties**.**

Some advantages of sol-gel method are:

A-can create a light coating-bone to give perfect adherence among the top coat and the metallic substrate

B- Low sintering temperature ranged from 200 ºC-600 ºC **(Ganguli, 1993).**

## 1.12.1 Dip coating technique

Dip coating is a method of surface coating by a fine film of required material, the samples is dipped in a solution contain the coating materials and then evacuated by a regulated speed and by planned temperature and climatic. This method is not difficult to achieve, economic and can adapt to variations of the sample surface **(Aksakal *et al.*, 2008; Nasir *et al.*, 2016)**. Essentially the dip coating method can be grouped into three steps **(Schneller *et al.*, 2013)**:

- **Immersion and dwell time**: the dipping of the samples inside the previously prepared solution then appropriate dwell time, to have enough interaction between the samples surface and coating solution.
- **Deposition and drainage**: through pulling of samples in upward direction a delicate coat of prepared solution is dragged beside deposited film, likewise overflowing fluid will leak from the sample surface.

- **Evaporation**: solvent evaporation from the liquid will form a sedimented soft film, the film can be improve by heat (drying). After that heat treatment to fire all organic chemical residual from the sample and to enhance crystallization.

## 1.13 Bone graft

Bone grafts are applied as a scaffold and filler to promote wound healing and to facilitate bone formation. These graft have no antigen-antibody reaction and considered as bioresorbable material, the bone graft induce bone formation by acting as a mineral reservoir (**Kumar *et al.*, 2013).**

Bone grafting is surgical method used to restore a lost bone by substance derived from the host, a synthetic, natural substitute or artificial.

Grafting of bone is achievable due to the bone has the tendency to reconstruct entirely if add the gap into which it has to generate. It generally restore the graft material completely, as natural bone grow, producing new bone fully integrated (**Kumar *et al.*, 2013).**

**Classification of bone grafts based on material groups (Laurencin, 2006)**

a. Allograft-based bone graft includes allograft bone, applied in incorporation beside several substances or alone like (OrthoBlast, Grafton).

b. Factor-based bone graft recombinant growth factors are natural, used in combination with other materials or alone like transforming growth factor-beta (TGF-beta), platelet-derived growth factor (PDGF), bone morphogeneic protein (BMP) and fibroblast growth factors (FGF) .

c. Cell-based bone grafts cells are added onto a support matrix to form new tissue are mesenchymal stem cells or alone.

d. Ceramic-based bone graft substitutes consist of calcium sulfate, bioglass and calcium phosphate applied in combination or alone; like, (ProOsteon, OsteoGraf, OsteoSet).

e. Polymer‑based bone graft administers non-degradable and degradable polymers incorporation with other substances or alone, like, open Porosity polylactic acid polymer.

Four factor are essential for bone grafting success (bone regeneration): Osteoinduction, osteoconduction, osteogensis and osteointegration **(Greenwald *et al.*, 2001; Kneser *et al.*, 2002).** Oseoinduction is the ability of procedure or chemical to enhance bone formation by recruitment of osteoblast and differentiation, conversion of mesenchymal cell in to osteoblast **(GPT, 2017).** Bone morphogenic protein is the most widely studied type of osteoinductive material **(Klokkevold *et al.*, 2002).** Osteoconduction is the method by which bone evolve on a scaffolding or on surfaces that is initiative for bone deposition: which is static method **(GPT, 2017).**

Osteoconductive and osteoinductive bone graft material have additional advantage beside scaffold for currently existing osteoblast which initiation the formation of new osteoblast **(Boyan *et al.*, 2000).** Osteogensis is the production of new bone through osteoblast within bone substitute material **(Scaglione *et al.*, 2014).** Osteointegration is direct contact of graft material to living bone **(Boyan *et al.*, 2000).**

Osteogenic cell inclusion into graft now still difficult method, due to less cell remain during transplantation. Most osteogenic cell appeared in the graft are enrolled there from the host bed by osteoinduction. This confusion is obvious issue when the bed viability is compromised, like violently fibrotic defect. So the production of bone graft substitute or bone graft that can done separately from the host bed condition is favorable **(Sutherland *et al.*, 2005).**

The space creating ability of bone graft material should be regarded alone from bone remodeling and formation appearing in almost early stage after new bone formation. Pressure from adjacent tissue like gingival flap or mucosa or Schneider membrane as a consequence of tissue retraction is one of the factor that

decreasing the quantity of packed bone particles or substitute granules during bone generation **(Tejero, 2001).**

## 1.14 Bone graft with implant

Surgical procedure like sinus lift, immediate insertion in extraction socket, bone graft can be used along with implant, the bone graft will support and preventing sinus membrane collapse, preserving its space and eventually promote bone formation **(Pettinicchio *et al.*, 2012).**

Defect with critical size usually not heal spontaneously unless placement of graft during the healing period (**Schmitz and Hollinger, 1986).** Thus bone augmentation is recommended in the gaps wider than 2 mm left between the coronal neck and the socket wall of the implant during implant placement immediately. All grafts have their exclusive properties due to which they are applicable in various conditions. Autogenous grafts are gold standard in reconstructive surgery, have the disadvantages of limited available bone volume and donor site morbidity. Dense, crystalline hydroxyapatite (HA) is applied in support region of the denture to keep contour and volume and not in socket preservation as it is almost nonresorbable and three time as the hardness of bone. While β-tricalcium phosphate (β-TCP) is regarded applicable in defect with contained type due to the desirable substitution rate in a standardized bone defect, its application as volume expander around autogenous onlay blocks is questionable due to the same reason and onlay grafting. Anorganic bovine bone (ABB) shows osseous integration in mature bone but no evidence of substitution of graft particles, thus showing partial nonresorbability. Hence the choice of graft material is depend on its and macromolecular biochemical profile and application **(Jensen *et al.*, 2006).**

It has been found that buccal gap grafting with implant insertion immediately leading to preserve soft and hard tissue dimension (**AlKudmani *et al.*, 2017).**

Bone graft insertion with implant simultaneously reduce healing interval with no risk of raising complication or minimizing the success rate **(Boronat *et al.*, 2010).** Primary stability during implant insertion has been improved with bone grafting technique **(Hegde *et al.*, 2016).**

### 1.14.1 Biochemical properties and structure of some graft material

**A-Autograft:**

Transferred and harvested from extra oral or intraoral to bone defected site with in the same person, autograft can be regarded as the most reliable source for osseous tissue regeneration. Autograft have many properties like osteoinductivty because of bone morphogenic proteins, osteogenicty and osteoconductivty **(Conrad *et al.*, 1995).** Autografts can be of three types: bone marrow, cancellous and cortical **(Khan *et al.*, 2005).**

**B- Allografts:**

Transferred from intertidally non-mach parts of the exact kind, such as from another person. They are obtainable in high volume for application and don't possess the conventional weakness related with autografts. Cortical and Cancellous allografts of different range of particle sizes are usually applied for bone remodeling method with reduced danger of disease infection because of virucidal tissue processing methods and the screening **(Block *et al.*, 2004; Sterio *et al.*, 2013).** Also, disease transmission and possibility of tissue contamination with new unknown pathogens due to some hazard as these may not be removed by current procedure of tissue processing and donor screening. no documented condition of prion disease transmission from bone allografts **(Aguzzi *et al.*, 1997).**

**C-Xenograft**

These are taken from the bone of non-humans of other species, their biomechanical properties and composition being almost identical to bone. Two illustrations of xenografts are used in dentistry 1) coral-derived bone substitutes having structure identical to that of human cancellous bone interconnected

macropores (200-600 μm) and 2) demineralized bovine bone grafts, osteoconductive and biocompatible. There are two types of demineralization:

a. High temperatures.

b. Chemical extraction of calcium and other minerals (**Jensen *et al.*, 2009**).

## D-Ceramic – based bone substitute

Ceramic-based bone grafts are artificial materials that have been generally applied to minimize the demand for autograft (iliac grafting). Ceramic origins are ironically linked preparations contain a set collection of bone graft material and inorganic**.** Ceramics diverge generally dependent on various in content, structure porosity, and manufacturing. Ceramic material contain different component like; calcium sulfate, hydroxyapatite and tricalcium phosphate. All Ceramics material has possible features of biodegradability, mechanical properties and different binding ability **(Berven *et al.*, 2001**).

Ceramic structure can be made to reduce inorganic content in the bone and are valid as porous or compact design. Porosity applied for mesenchymal cell rapid growth, differentiation and adhesion, into fully developed osteoblasts **(Khan *et al.*, 2005).** As the porosity of ceramics increased, the mechanical strength also increased and rate of resorption are compromised **(Berven *et al.*, 2001**)**.**

Optimal orifice scale has been ranged from 150 and 500 μm to raise biodegradation, bone ingrowth and interface activity **(Truumees *et al.*, 1999**)

Ceramics produce a considerable osteoconductive ground substance but with less osteoinductive properties. As consequence to that a promising arthrodesis ratio are considerably greater when applied with cell origin like bone marrow aspirate or local autograft. Ceramics are biodegradable; however, remodeling appear by a various method than conventional bone remodeling. Resorption of material by giant cells, rather than osteoclasts and resorption completely may not appear during the 10 years **(Gazdag *et al.*, 1995).**

Ceramic have many advantages compared to other bone graft material, do not produce inflammatory host response and they are biologically inert, besides that,

they can to be sterilized without lowering the danger for disease infection and loss of structural integrity. Ceramics can be molded and cut into different forms to precisely adapt to different conditions like those in inter body lumbar fusions and posterolateral. At last, the cost of ceramics is affordable when compared to other bone graft material **(Berven *et al.*, 2001; Khan, *et al.*, 2005).**

## 1.15 Egg shell as bone substitute material

A wide range of bone substitute or graft material used to correct bony defect in dental and orthopedic field. In oral implantology the demand for grafting is increased to improve the clinical outcome .Lately egg shell composed of CaCo3 (almost 93.7%) had shown to be a good bone graft material especially in maxillofacial surgery procedure due to its mineral composition and good mechanical properties **(Duporieux *et al.*, 1995; Duporieux *et al.*, 2000, Duporieux *et al.*, 2001).**

Egg shell thickness ranged between 200µm-400µm.The main parts of egg shell is described as follow: mineral (96.1%), protein (3.3%), water (1.6%) of the component. The main element of mineral part is calcium carbonate ($CaCo_3$) about (93.7%) then calcium triphosphate and magnesium carbonate **(Duporieux *et al.*, 1995).**

The protein content of egg shell could be vary according to different structure. Organic matrix of egg shell composed at least 70% protein and 11% polysaccharide **(Duporieux *et al.*, 1995).**

The calcified egg shell consists of an organic ground substance establishing of almost 3% of the eggshells total weight. Moreover the calcified egg shell contains proteoglycans and proteins such as (ovalbumin, ovocleidin-116, lysozyme, ovocalyxin-32, ovocleidin-17 and osteopontin), in which some of these are able to change eggshell calcite the rate of precipitation and crystal morphology (**Pines *et al.*, 1995; Panheleux *et al.*, 1999).**

Egg shell powder has positive effect on bone metabolism and its safe and biodegradable, these characteristic made egg shell good candidate for bone grafting **(Durmus *et al.*, 2008).**

Egg shell is used as alternative to bone substitute material as has been proven in vivo studies **(Park *et al.*, 2009).** Initial studies have concentrate on biocompatiblty, biological behavior of egg shell and bonding ability to bone **(Duporieux *et al.*, 2001).** No inflammatory or toxicity effects of this natural product are documented or confirmed, beside to its biocompatibility, eggshell is more than only a type of calcium carbonate supply. The organic components of egg shell has unique importance regarding: cell migration, osteointegration, cell proliferation and important stages for bone formation **(Neunzehn *et al.*, 2015)**.

$CaCO_3$ derived from egg shell has been described to be effective as a osteoconductive and biocompatible biomaterial in many experimental animal studies **(Dupoirieux *et al.*, 2001; Durmus, *et al.*, 2003).**

It was studied that most defects filled by egg shell powder cured with fibrous union **(Dupoirieux *et al.*, 1995; Dupoirieux *et al.*, 2001).**

$CaCO_3$ mostly reported as fully resorbable and biocompatible therefore its useful in bone curing as no carbonate transition to the surface to begin bone generation (**Yukna *et al.*, 1998; Guillemin *et al.*, 1987**)

The egg shell derived component like bioresorbable carbonate which is the beginning material for generation of bone substitute substance, also it has the advantage of biodegradability and availability. The clinical application of using ceramic hard tissue reconstruction by using these materials began in the late eighteenth (**Barrere *et al.*, 2003; Tas *and Aldinger* 2005).**

The application egg shell derived bone material for healing purposes in osseous defects of rat calvaria using modified surface of egg shell

particles have acceptable osteoconductive feature, with maximum level of straight bone apposition on their surfaces and new bone formation **(Park *et al.*, 2008).**

Egg shell contain large amount of calcium, so there is ability to use egg shell as dentin bridge former when its placed on exposed pulp **(Salah *et al.*, 2018).**

# Chapter Two

# Materials and Methods

# Materials and Methods

## 2.1 In vitro experiments

### 2.1.1 Materials and instruments

1. Commercially pure titanium rod (CpTi) rod grade (II) (29mm diameter, 31cm length) (Orotig S rl EU Company, Italy).
2- Nanocalcium sulfate hemihydrate (Orthogene cop. USA) (Exp.date 2020).
3. Egg shell.
4. Ethanol absolute 99.8%. (SIGMA ALDRICH, Germany) (Exp.date 2019).
5. Distilled water (china).
6. Glass petri dishes, glass beakers and test tubes (USA and China).
7. PVA (Poly vinyl alcohol), (Sigma Aldrich, Germany) (Exp.date 2019).
8. PVP (Polyvinylpyrrolidone) (Sigma Aldrich, Germany) (Exp.date 2020).
9. Phosphorous pentoxide $P_2O_5$ (Emphos PS-21A, Witco) (Exp.date 2021).
10. Silicon carbide paper (china).
11. Nitric acid ($HNO_3$) and Hydrofluoric acid (HF) (Biosolve, UK) (Exp. date 2022).
12. Heat shrinking plastic wire sleeve (China).
13. Desiccator (Grag, India) .
14. Argon gas (Iraq).
15. Acetic acid 2% (Scharlau S.L., Spain) (Exp.date 2019).

### 2.1.2 Equipment

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◦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. Glass containers (china).
11. Lathe machine (Bantam, Italy).
12. Rotative grinding and polishing machine.

## 2.1.3 Sample preparation

Commercially pure Titanium (grade II) was cut into small circular discs (10 mm diameter and 1 mm thickness) with a lathe machine then used as the substrate for coating, as shown in (**Figure 2.1, Figure 2.2 and Figure 2.3).**

These discs 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 discs were cleaned by ultrasonic bath of ethanol used to get rid of debris and contamination for 15 min, **(Figure 2.2)**, followed by 10 min in distilled water bath **(Figure 2.3)**. After that the specimens left to dry at room temperature **(Shukur, 2014).**

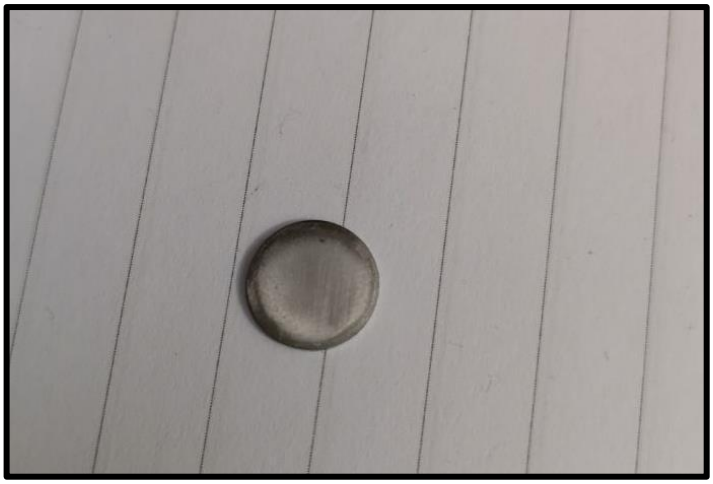


**Figure 2.1: Commercially pure titanium disc.**


**Figure 2.2: Ultrasonic cleaner.**

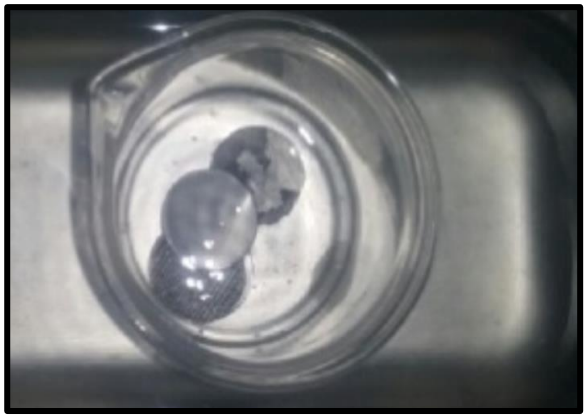


**Figure2.3: Cleaning of discs in distalled water.**

## 2.1.4 Pilot study

## 2.1.4.I. Solution preparation

## A. Preparation of Nano calcium sulfate coating solutions

Three coating solution of nano calcium sulfate were prepared according to manufacture instruction plus different additive for each solutions as the followings:

1-The first solution was prepared by the addition of nano calcium sulfate **(Figure 2.4 A)** (10 g) to 50 ml NaCl plus (0.01g) of $P_2O_5$ in glass container and heating at 45 ºC on hot plate stirrer for half an hour, to get a homogenous solution **(according to manufacture instruction).**

2- A second solution was prepared by addition of PVA (0.5g) to solution of nano calcium sulfate (10g) in the 50 ml of NaCl. The temperature was maintained at approximately 45 ºC, the mixture left over a stirrer for half an hour to gain homogenous solution.

3-The third solution was prepared by the same procedure of the second solution except the addition of (0.5g) of PVP to the PVA (0.5g).

**2.1.4.2 Disc coating:**

The coating was done by using a electronic device locally manufactured for dip coating that hold the discs by braces and contain time regulator to control the immersing time of the discs in the mixture solution for (30 sec, 60sec ) four layer of coating film each layer dipped for 60 sec and withdrawn with a well defined with drawl speed (10 sec), dried for one minute at room temperature and then returned to its coating solution.

Disc coating was made by immersing the discs in nano calcium sulfate solution 60 sec four times. After dipping procedure, each coated sample was left for 24 hour at room temperature for drying.

For the solution that used $P_2O_5$ as a binder, the coating layer was detached easily and didn't bind with Cp Ti substrate so it was cancelled from this study.

The optical microscope and AFM was used to evaluate the distribution and homogeneity of the coated film for three previous types of solutions and two dipping times.

The final best result was shown in the third solution that contain the combination of 10 g nano calcium sulfate + 50ml NaCl + (PVA 0.5g +PVP 0.5g) and 60 sec four times dipping , where the obtained coated layer uniform and with minimum noticeable cracks and lowest lost particle of the coating material, and highest roughness value (3.28 nm)**(Figure 2.4 B, Figure 2.5, Figure 2.6 and Figure 2.7).**

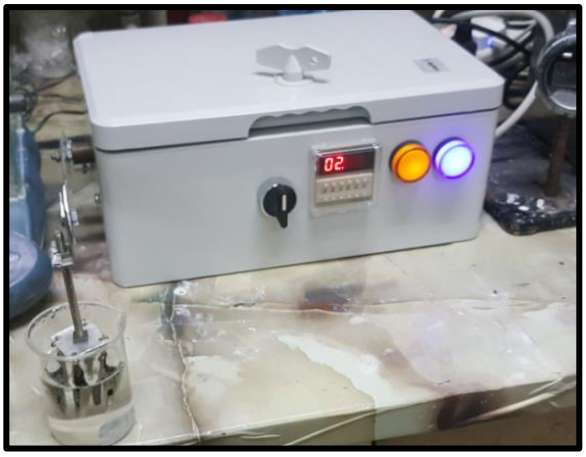


**Figure 2.5 Coating device.**

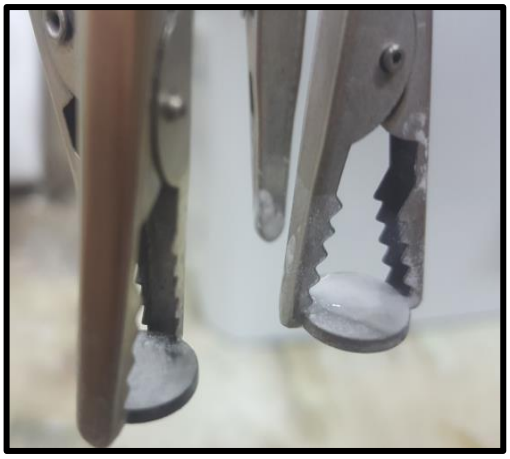
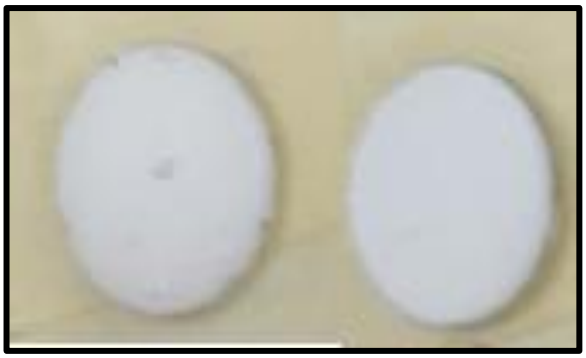
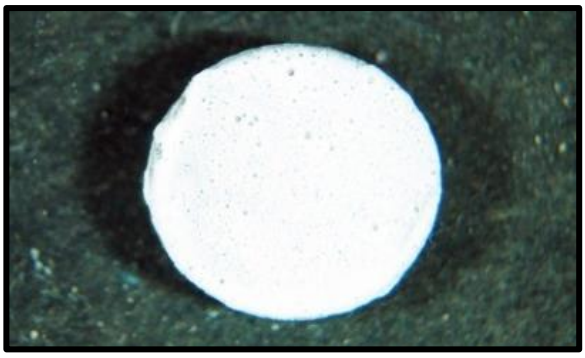


**Figure 2.6 Braces holding the discs.**

(A) (B)

Figure 2.4: Solution preparation: (A)Nano calcium sulfate powder,(B) Prepared solution of nano calcium sulfate.





**A** **B**

**Figure 2.7 Coated discs.**

### 2.1.4.3 Preparation of egg shell powder as bone substitute material

Egg shell were purchased from a local market, the egg were rinsed with distilled water broken and emptied of white and yolk, the membranes were peeled off manually and the egg shell were ground to powder with a porcelain pestele and mortar and kept in a desiccator to avoid humidity in egg shell powder, the powder was saived to various sizes which are (less than 50, then 50, 75,150)µm (**Dupoirieux, *et al.*, 2001) (Figure 2.8 and Figure 2.9).**

**Figure2.8: egg shell.**

**Figure 2.9: egg shell powder.**

## 2.1.4.4 Heat treatment

Heat treatment of the nano-calcium sulfate was carried out for densification using carbolated furnace (tube furnace) under the presence of inert gas (argon) to avoid oxidation of titanium disc, the sintering of nano-calcium sulfate coated disc was tried at different temperature (200, 400, 550, 600 ºC), for 2hour **(Harle *et al.*, 2006)**, the most appropriate heat treatment was (550 ºC) for 1 hour.


**Figure 2.10: Carbolite furnace**

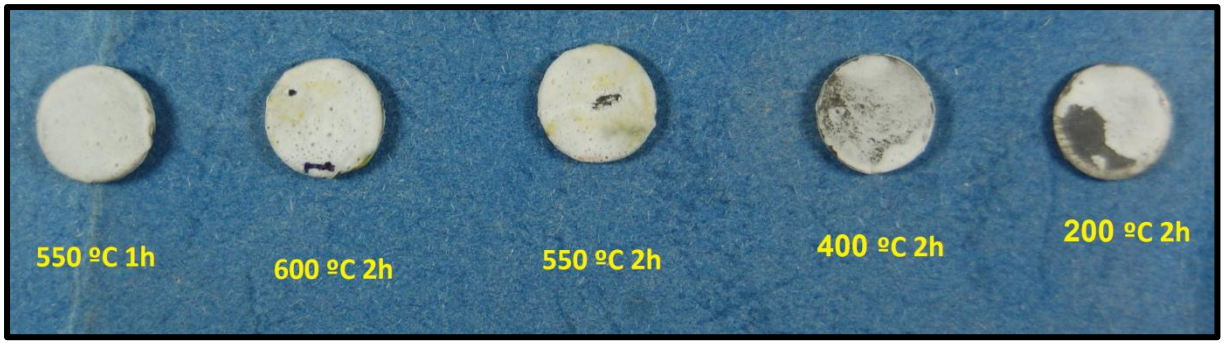



**Figure 2.11: Heat treatment at different temperature**

## 2.1.5.5 Coating characterization

### A- Thickness measurement

All coated discs thickness were calculated by using microprocessor coating thickness gauge (Erchsen minitest 3000), (**Figure 2.12**) this investigation is regarded as non-destructive procedure, considering the coating thickness analysis. The thickness measurement is performed by choosing three dots on the coating layer, the first dot on the periphery of the disc and the remaining two dots on the same line toward the center of the disc then the average of these measurements was calculated which was considered the thickness of coated disc.

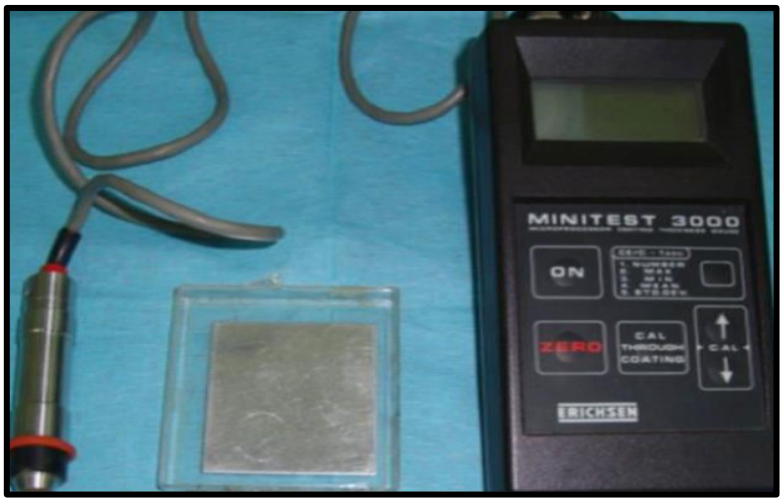



**Figure 2.12: Digital gauge for coating thickness.**

## B- Microscopical examination

The examination of the surface, along with manifestation of the coating was done by optical microscope, all coated disc were tested by using optical microscope (Nikon Type 120, Japan optical microscope) (**Figure 2.13** ), this optical microscope have digital camera type DXM 1200 F, under various magnification power (100µm, 250µm).

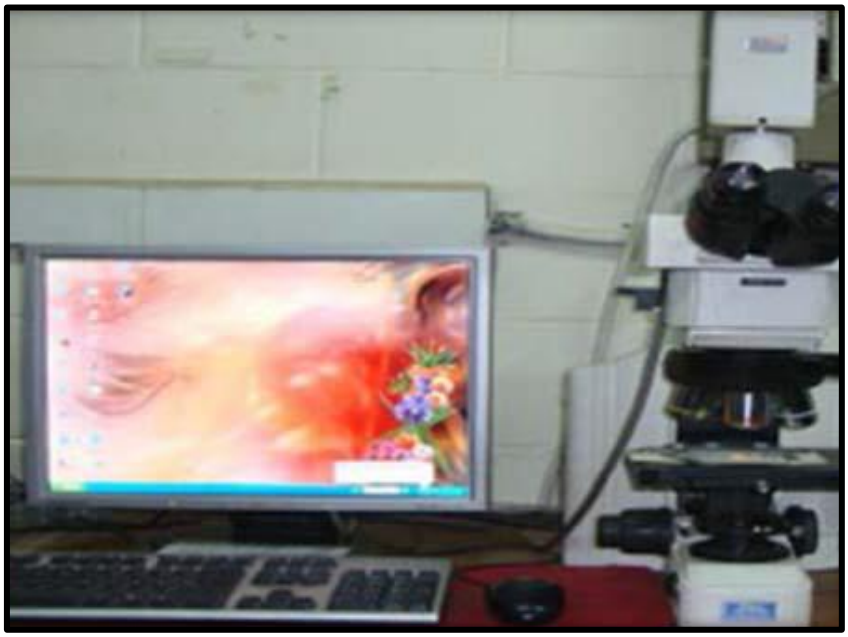


**Figure 2.13: Optical microscope with digital camera.**

## C-Atomic force microscopy

Atomic force microscopy **(**AFM) is a very high resolution type of scanning probe microscopy (SPM) **Figure 2.14**). AFM can demonstrate a resolution (on the order of fractions of a nanometer) better than the optical diffraction limit in more than thousand times **(Ashton, 2013)**.

Analysis of nano surface roughness and granularity distribution of coating film for dip coated samples (including the pilot study samples) were examined by using atomic force microscopy.

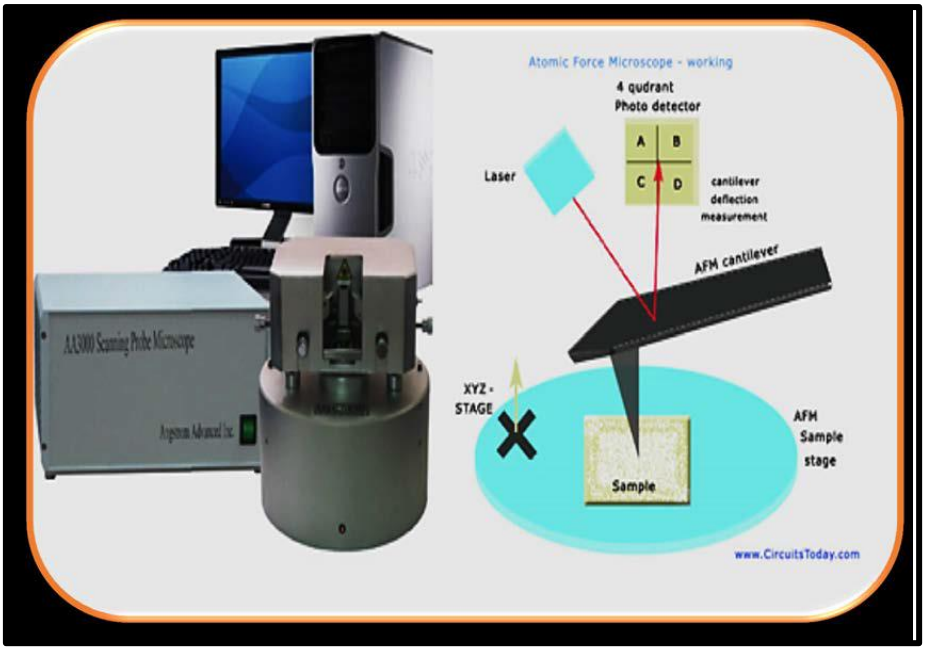



**Figure 2.14: Scanning probe microscopy.**

## D- X-Ray diffraction analysis

Phase analysis was employed on samples for dip coated nano calcium sulfate and two sample of egg shell powder (the larger particle size and the smallest one). Phase analysis was employed with X- ray diffractometer by using Cu Kα radiation as shown in **(Figure 2.15)**. The 2Θ angles were swept from (20-60) ° in step of one degree. The peak indexing was carried out based on the joint committee on powder diffraction standards (JCPDS) of the international centre for diffraction data.

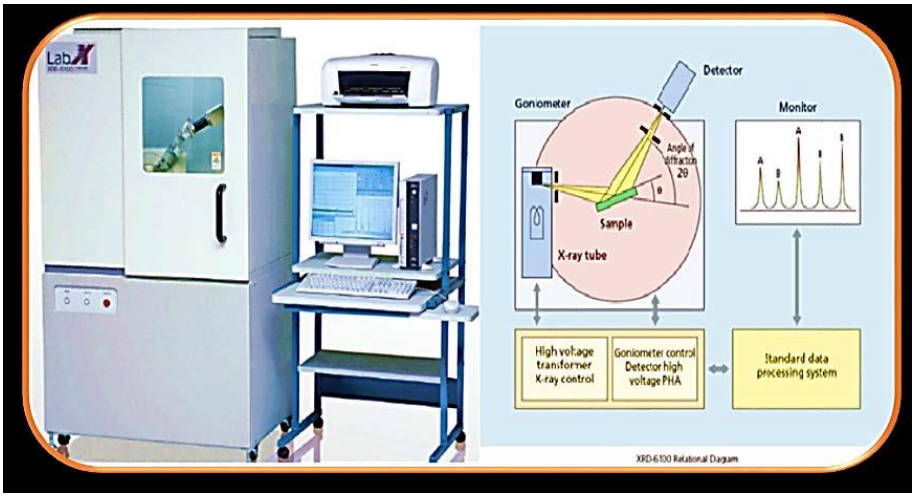



**Figure 2.15: X-ray Diffractometer device**.

## D- Scanning electron microscope (SEM)

Morphological analysis was employed to uncoated and dip coated nano-calcium sulfate discs, to produce better images, each sample was inserted in ion sputter to get gold ion sputtering **(Figure 2.16)** and then was imaged by using scanning electron microscope (SEM) **(Figure 2.17)**. In SEM technique, an electron beam is scanned over the surface of the sample. The electron beam induces a larger focus depth than a beam of a regular light. Therefore, very high resolution images can be recorded **(Reed, 2005; Goldstein *et al.*, 2017).** This device is therefore used for studying the morphological and topographical surface characteristics.

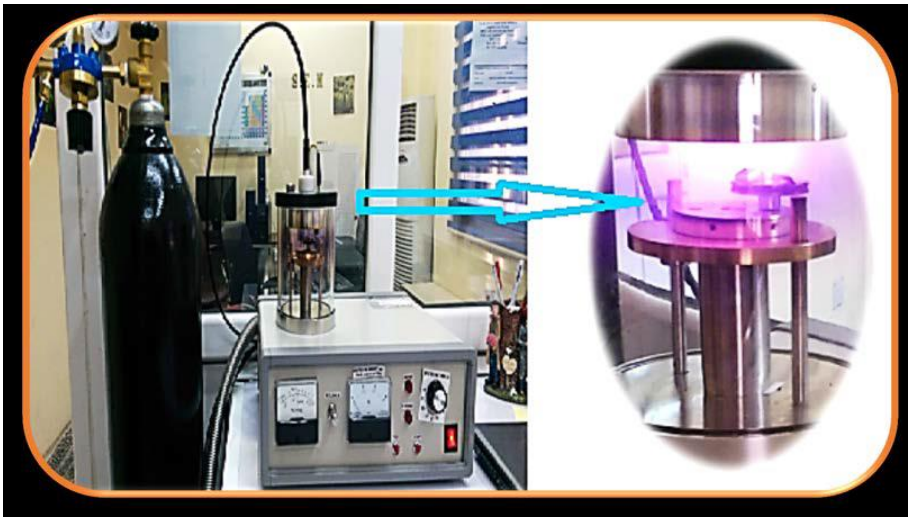


**Figure 2.16 Ion sputtering.**

**Figure 2.17: Scanning electron microscope.**

**F- Energy-dispersive X-ray spectroscopy (EDX) analysis**

Energy-dispersive X-ray spectroscopy (EDX) analysis was made for the coated samples within the SEM instrumentation. In this analysis, the X-ray photons emission formed as a result of the excitation and relaxation of sample atoms when the incoming electron beams interact with it. These emitted X-ray photons considered as a characteristic feature to the elements produced them. Therefore, both quantitative and qualitative elemental analysis can be done using the EDX analysis **(Goldstein *et al.*, 2017)**.

## G-Micro-hardness measurement

Vickers micro-hardness tester **(Figure 2.18)**, was used to measure the micro-hardness of the prepared small circular discs substrates. (10mm in diameter and 1mm in thickness) four groups were tested, the first group contains uncoated samples, the second group contains specimen that coated by 2 layer each one (60 sec), the third group specimen that coated by 3 layer, each one (60sec) and the fourth group contains specimen coated with $4^{th}$ layer of (60 sec).

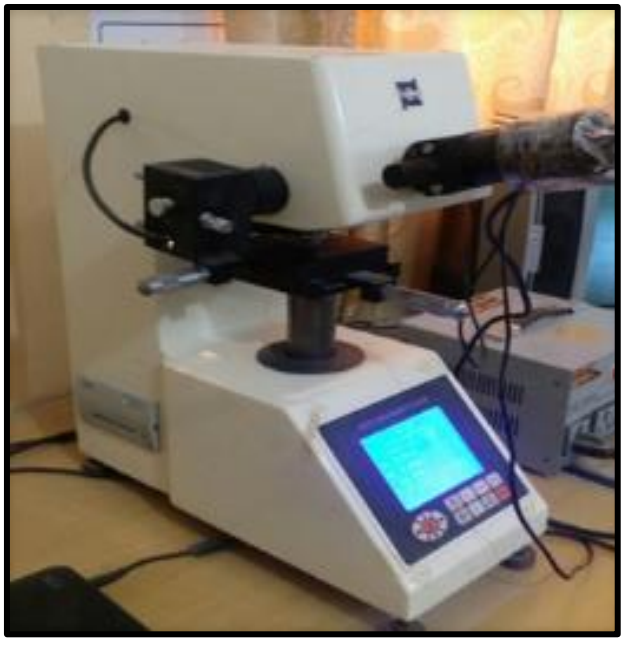


**Figure 2.18: Vicker micro-hardness test.**

## H- Fourier transfer infrared 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 and De Hasseth, 2007).**

## 2.2 Implant preparation

### 2.2.1Methods

For preparation of the implant screws, Cp Titanium rods (grade II, 6mm diameter) were used. The chemical compositions of these rods are demonstrated in **(Table 2.1)**.

**Table 2.1: Titanium rod chemical composition.**

| Elements | Titanium | Iron | Carbon | Others |
|---|---|---|---|---|
| **Weight** | 99.3% | 0.3% max | 0.08%max | 0.32% max |

Eighty screws were prepared by using a lathe-cut machine having a cutting head made of titanium carbide. Each screw was (8) mm in length (the threaded part was 6mm, the non-threaded part was 2mm) and the diameter of the screw was (3) mm. A slit with (1) mm width and (1.5) mm depth was located centrally in the screw head to fit a screw-driver and a torque meter during the insertion and removal process. The implant design is shown in **(Figure 2.19).**

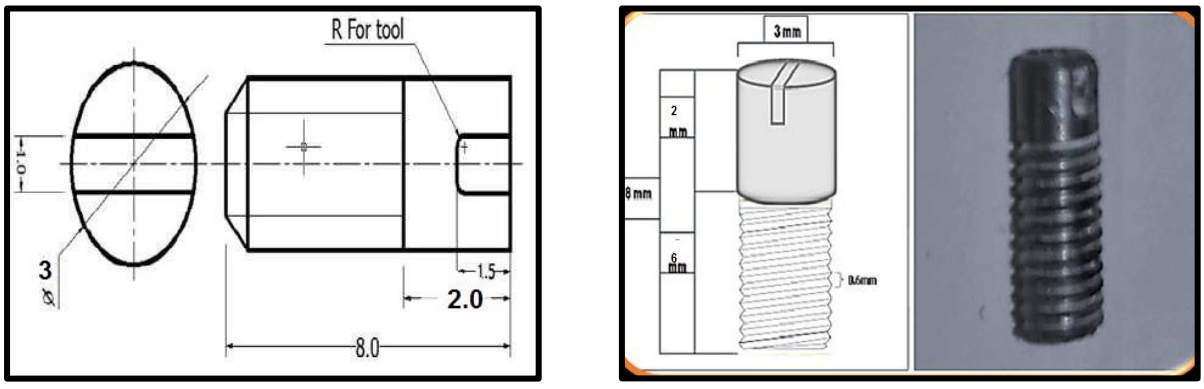



**Figure 2.19: Implant design.**

The screws were cleaned first to remove surface contaminations and to get a clean and uniform surface by using a solution consisting of a mixture of acids and distilled water (30% HNO3, 10% HF, and 60% $H_2O$) **(Lausmaa, 2001)**. Later and by using ultrasonic cleaning device, an ultrasonic bath of ethanol absolute (≥ 99.8%) was used to remove any ruminant debris, contaminations and acids from all screws. This process was performed for up to (15) min and then dipped in distilled water bath for (10) min. At the end, all of the screws were left at room temperature for dryness 24 hours (**Shukur, 2014).**

Concerning implant coating, the proximal unthreaded portion of the implant must be sufficiently smooth to minimize adherence of bacteria that can cause an adverse mucosal tissue reaction as a result of crestal bone loss **(Schupbach *et al*., 1994)**. Therefore, and for better standardization as well as to prevent any effect of coating on this unthreaded area on the future histological and torque meter results, only the (6) mm threaded part of the screw (which would be completely introduced into the bone tissue of the rabbit tibia) must be coated. However, the removal of coating layer from this unthreaded area of screw can affect the remaining coated layer (on the threaded part) by cracks or loss of part of the coating layer. For these reasons, the unthreaded part of each screw was warped by tight heat shrinking plastic wire sleeve before coating procedures **(Azzawi et al, 2018) (Figure2.20)**.

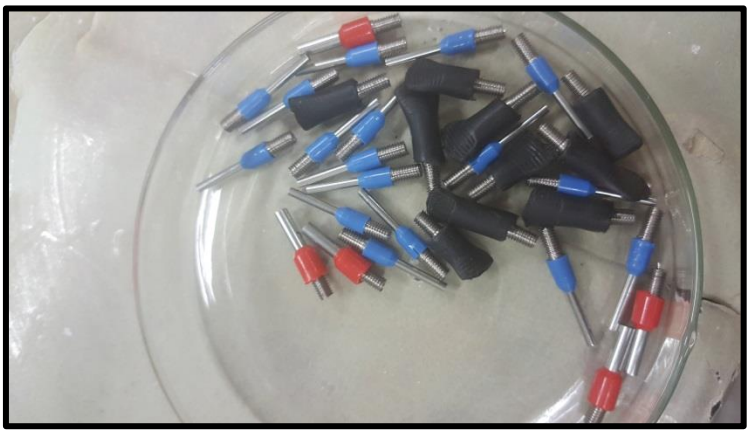


**Figure 2.20: Covering the head of screws.**

### 2.2.2 Dip coating technique

A total of 60 screws were used for the dip coating technique. Each screw was dipped in the prepared nano calcium sulfate solution for (60) sec four times and then with drawn with well defined withdrawal speed by coating device **(Figure 2.21).**

**Figure 2.21: Dip coating procedure.**

After dipping process, the samples were left for (24) hours in air for drying. Then, the sleeves were removed from the screws. For coating densification, a tube furnace was used for sintering of the coated screws. The heat treatment was done at a temperature of (550) °C for (1) hours **(Figure 2.22 and Figure 2.23).**

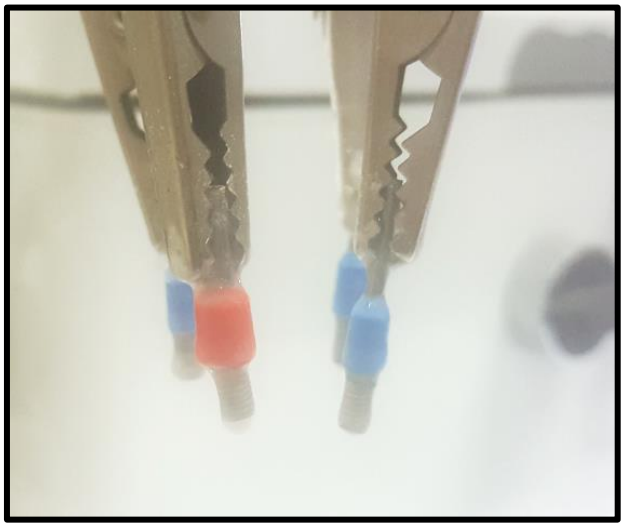


**Figure 2.22: Screw dryness.**

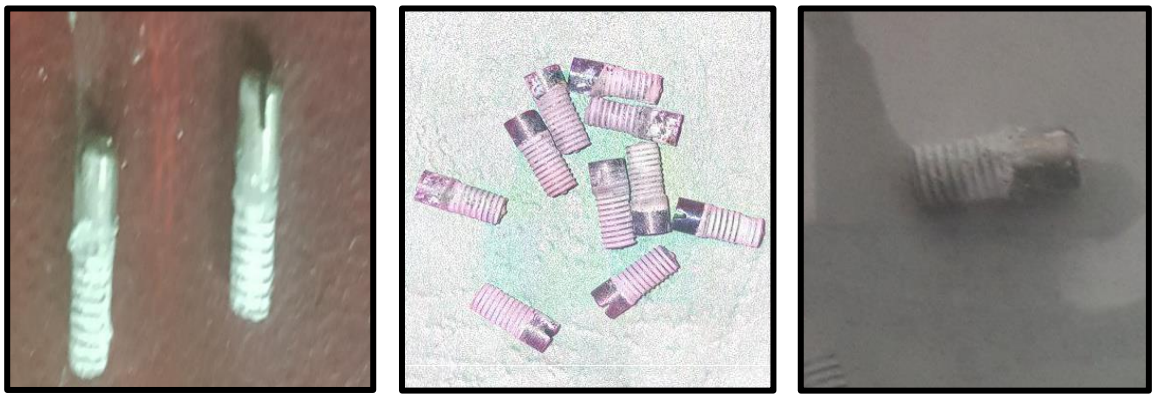


**Figure 2.23: Screw after heat treatment.**

## 2.2.3 Sterilization:

Each screw was kept in single airtight sheets before sterilization (**Figure2.24)**. Gamma radiation dose of 2 mega rad using gamma cell 220 with a CO60 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 min **(Figure 2.26).**

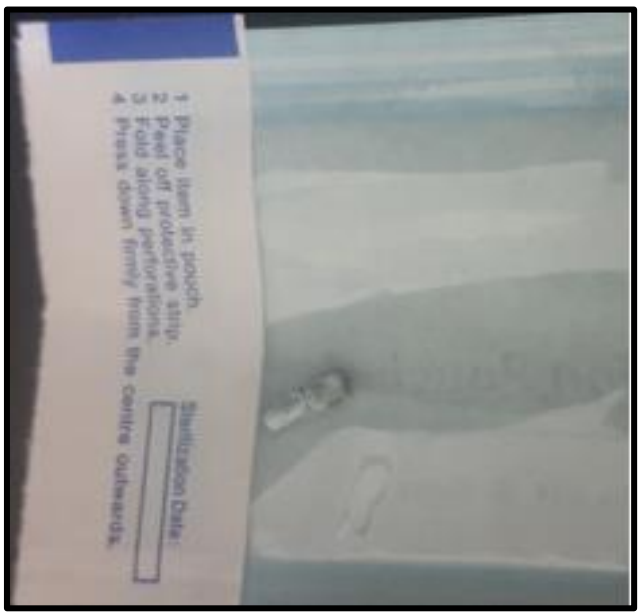



**Figure 2.24: Coated sterile screw.**

### 2.2.4 Sample grouping:

The total 80 screw were organized into 4 categories according to the test performed.

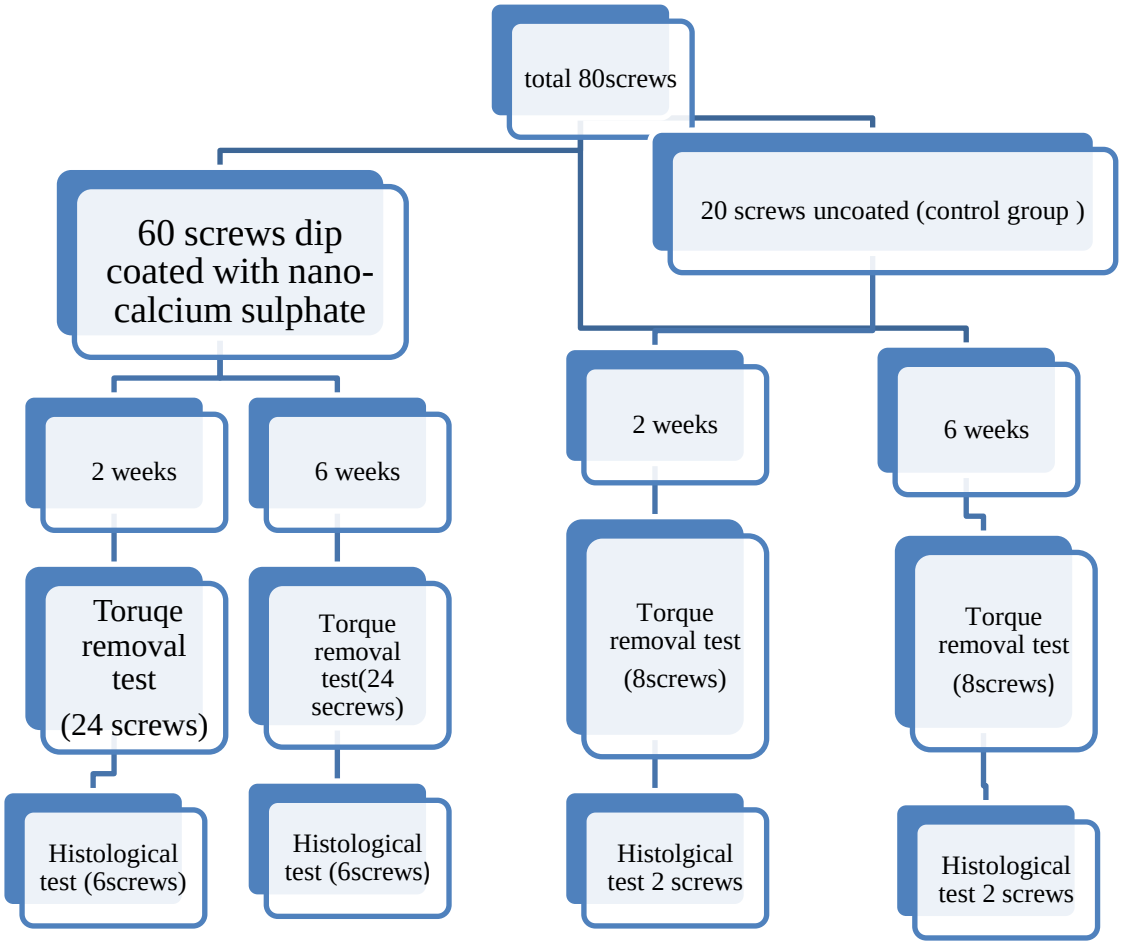



**Figure 2.25 Sample grouping**

### 2.2.5 Study design

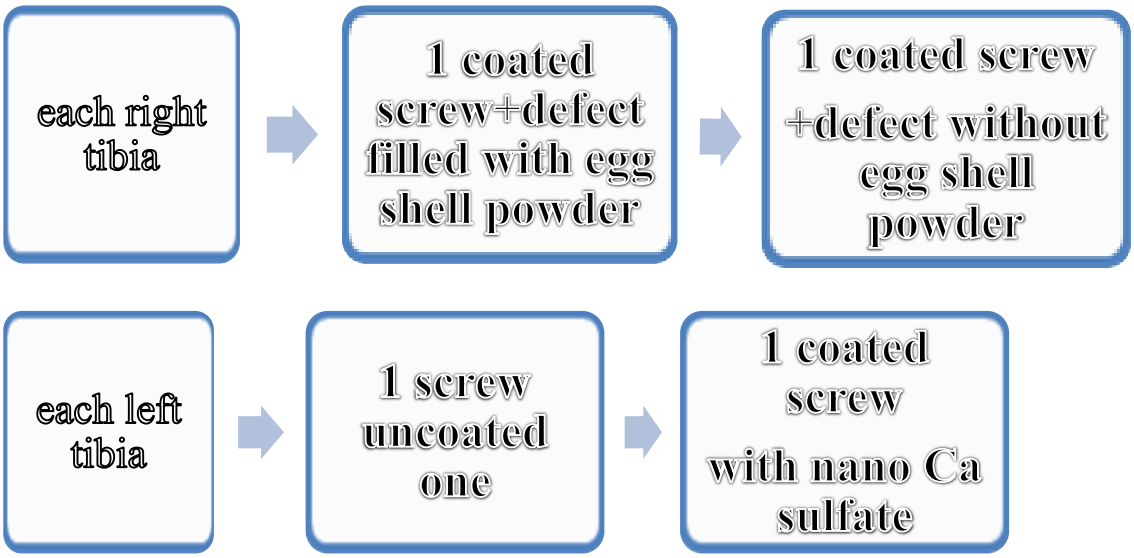



**Figure 2.26: Implant design**

## 2.3 In vivo experiments

### 2.3.1 Materials and instruments

#### 2.3.1.1 Experimental animals and design

Twenty adults male Newzeland rabbits were employed in this study, they all kept in special cages (40×60×70 cm) in the animals' house of Veterinary Medicine College /University of Baghdad, they kept in these cages for at least two weeks for daily inspection and well adaptation, the water was free and the food is a mixture of pallets and green grass, they prophylactic against most of the internal and external parasites by single dose (0.01 mg /kg Body weight ) of ivermectine **(Figure 2.27).**

**Figure 2.27: Experimental animal.**

## 2.3.2 Surgical procedure and implantation:

### 2.3.2.1 Materials and instruments (Figure 2.28)

1. Alcohol 70% (Al kindi –Iraq) (Exp.date 2021).
2. Anesthetic solution (Ketamine 100 mg (10%) Holland) and (Xylazine 20mg 2% Belgium) (Exp. date 2020).
3. Catgut suture. (3/0 DyNEK sutures, Australia) (Exp. date 2020).
4. Cotton and gauze (china).
5. Disposable non-woven bed covers (China).
6. Disposable syringes (china).
7. Drills (Densply, Maillefer, SWISS)
8. Iodine 10% (Lebanon) (Exp.date 2019).
9. Ivermectin 10mg (Syria) (Exp.date 2019).
10. Long-acting oxytetracycline (Kepro Oxetet 20%, Holland) Exp.date2021).
11. Needle holder (Germany).
12. Normal saline solution (0.9%Haidylena /Egypt) (2020).

13. Occlusal X-ray film (Kodak).
14. Scalpel with blades (Germany).
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-2020.)
21. Topical aerosol (spray) oxytetracycline (OTC, Iran-2021).
22. Tissue forceps **(**Faulhaber, Germany)

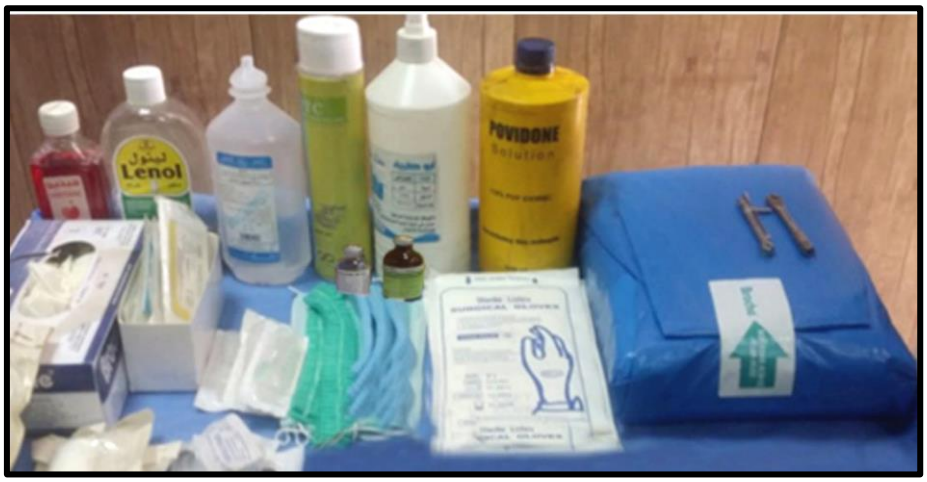



**Figure 2.28 Materials used in surgical procedure.**

### 2.3.2.2 Equipment

1. Autoclave (Woson medical instrument co, S1104BOO16W.China).
2. Balance (1-50 g) (China).
3. Engine (ASSISTANT, CNS INDUSTRIE srl. Via Aquileja 43/B, 20092 cinisello, Balsamo MI- ITALY) with hand piece **(Figure 2.29).**
4. Conventional X-ray machine (TOSHIBA, Japan).
6. Digital tourqe meter (TQ8800) (Dentium, Korea) (**Figure 2.30**)

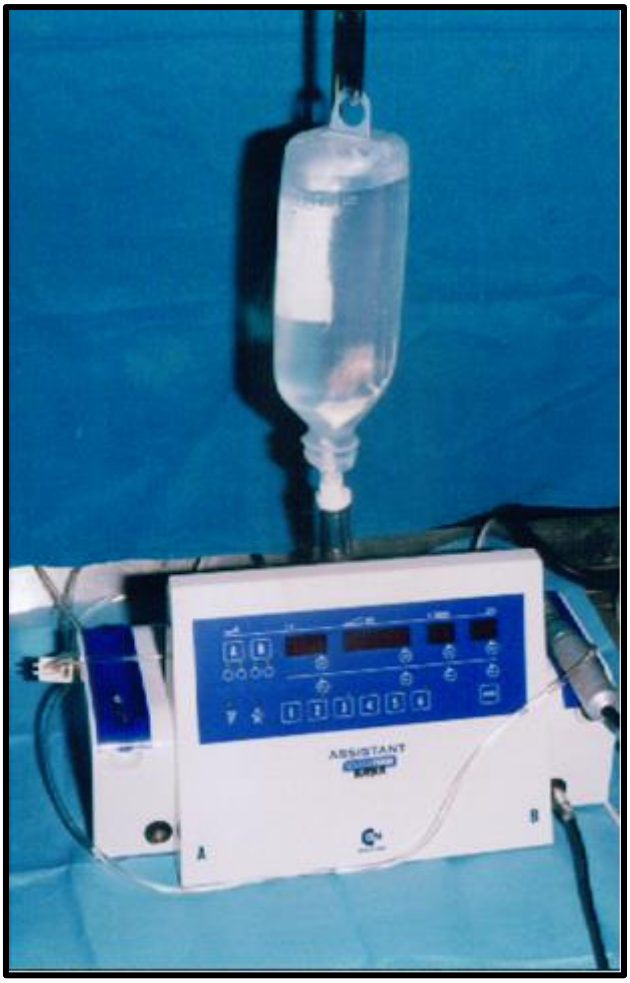



**Figure 2.29: Assistant engine used in surgical procedure.**


**Figure 2.30 Digital torque meter.**

### 2.6.2.3 I-Pre-operative preparation

All instruments and towels were autoclaved at 121 ˚C and for 30 min. Each animal was weighed before operation to determine the required dose of anesthesia and antibiotic, the site of operation prepared by clipping the limb hair shaving the medial aspect of the tibial bone of both hind limbs, wash the surgical site by tap water and surgical soap, then disinfect the area with 70% alcohol. The rabbits off food (24) hours prior to operation while the water (2) hours, the body weight measured by baby balance for proper anesthetics doses calculation {(body weight × standard anesthetic dose) / drug concentration} **(Refaat *et al.*, 2016).(Figure 2.31)**

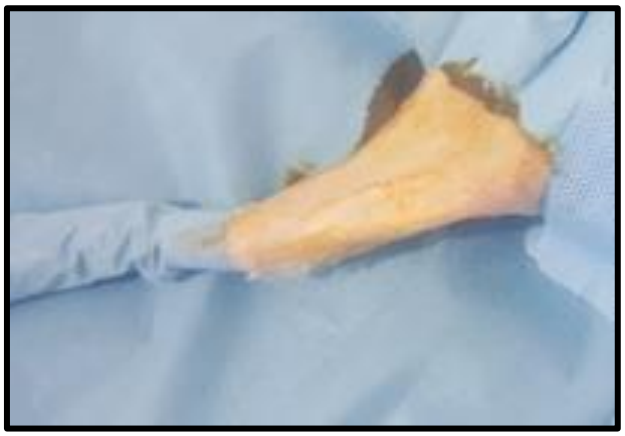


**Figure 2.31 Shaved skin.**

### II-Surgical technique

The general anesthesia was induced by intramuscular injection of xylazine hydrochloride 2% at dose 17 mg/ kg.BW and ketamine hydrochloride 10% at dose of 30 mg/ kg.B.W. respectively, after the induction of general anesthesia the operated rabbit lie down on the lateral recumbence with the medial side of tibia oppose the surgeon. The length of skin incision (3cm) which was done at the third proximal of the medial aspect of the tibia, the under lined soft tissues were bluntly dissected then the periosteum sharply dissected **(Figure 2.32).**

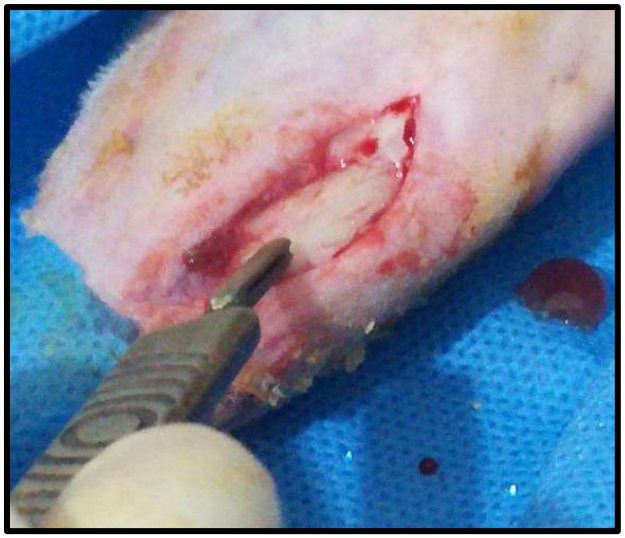


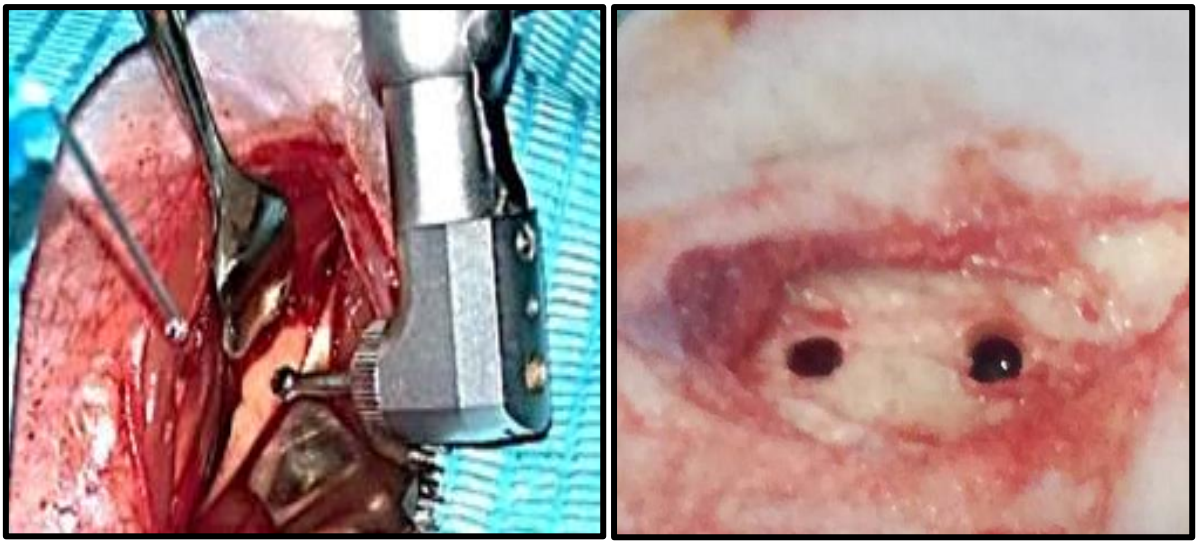
**Figure 2.32 Skin incision.**

The holes were drilled by the hand piece of assistant engine by intermittent and continuous cooling with irrigated normal saline, a mark about 6mm on the bur to determine the depth of the hole of the screw.

Two holes of 2.5 mm diameter with 1cm apart were prepared . The sterilized uncoated screw was inserted in the upper left hole, and the sterilized screw coated with nano calcium sulfate was inserted in the lower left hole.

A screw driver was used to seat both screws completely in its bed.

The torque meter was used to check the final insertion of the screw in bone tissue and to measure the insertion torque force which ranged from (0.9 N.cm- 1 N.cm).



(A) (B)

**Figure 2.33: Preparation of hole in tibia (A) prepartin of the hole (B) holes in position.**

The same technique was repeated in the right tibia then marker was used to mark a 2mm on the bur that reinserted in the hole so that the end of the marker was with the level of the surface hole to create a 2 mm space or gap around the coronal part of screw **(Figure 2.34).** A screw coated with nano calcium sulfate was inserted in the upper right hole and the gap filled with eggshell powder using amalgam carrier for 2 times. In the lower right pore the same method was applied but the gap was not filled with eggshell powder just the coated screw. Penicillin powder was applied for both tibiae on the surgical site.

Suturing of the muscles was done with absorbable catgut **(Figure 2.35)** followed by skin suturing with silk suture **(Figure 2.36).** Topical antibiotic (tetracycline) was used by spraying the operation area.

Post-operative care was provided and a systemic antibiotic (Streptopen- IM, 0.5 ml/kg) was injected once daily for (5) days.

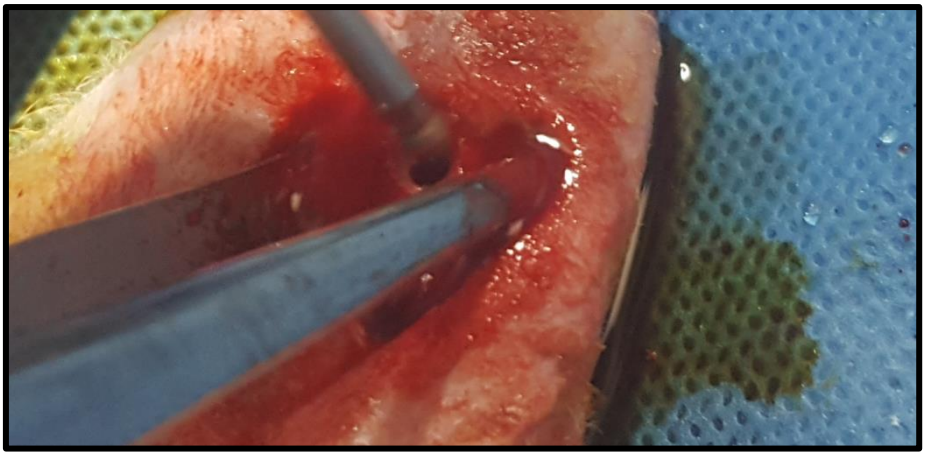


**Figure 2.34: Making a gap around implant position.**

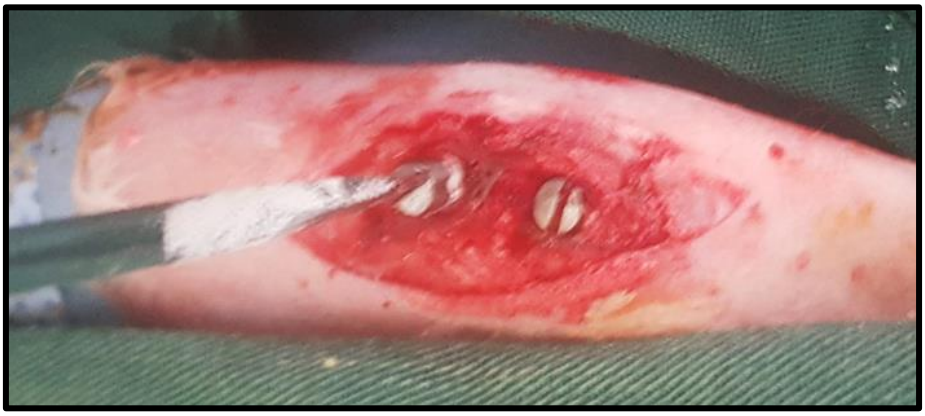


**Figure 2.35: Screw tighting by torque meter in the right tibia.**

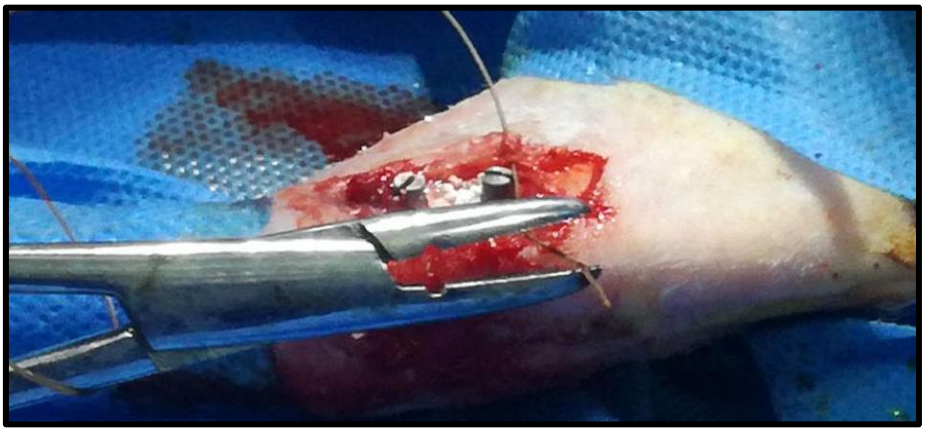


**Figure 2.36: Screws in position, penicillin application and suturing of muscle.**

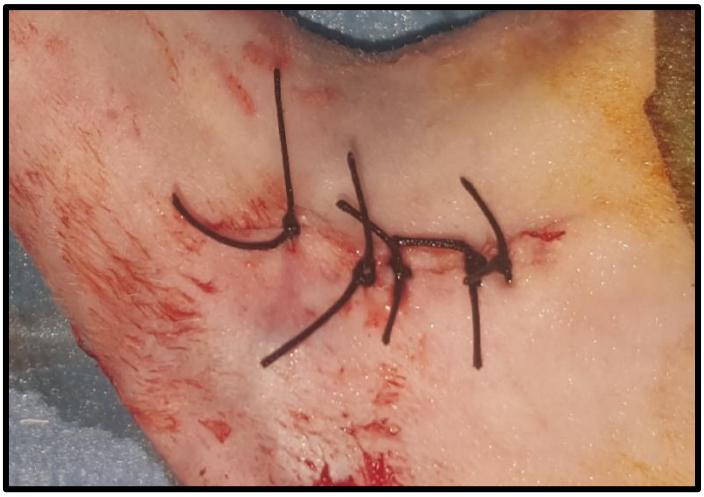


**Figure 2.37: Skin suturing.**

## III-The Radiological examination

The radiographic image were taken after 2 and 6 weeks, with light anesthesia to control the rabbits, with the medio-lateral view and the anterior – posterior view with the exposure factors (kilovolt) Kv=47 to 54, the ( millamper / second ) mAs = 2.20 to 4. The (focus film distance) F.F.D. = 30 cm. The x-ray machine type Eco ray (made in korea), the x-ray films processed in the dark room using automatic processor **(Figure 2.38)**

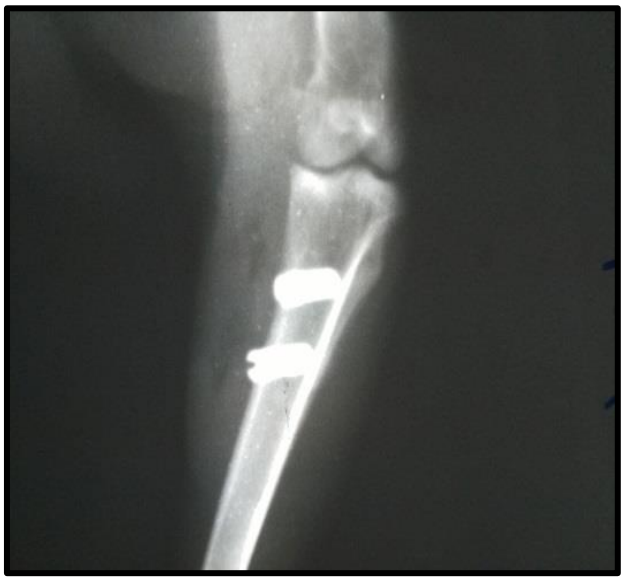


**Figure 2.38 Right tibia after 2 weeks.**

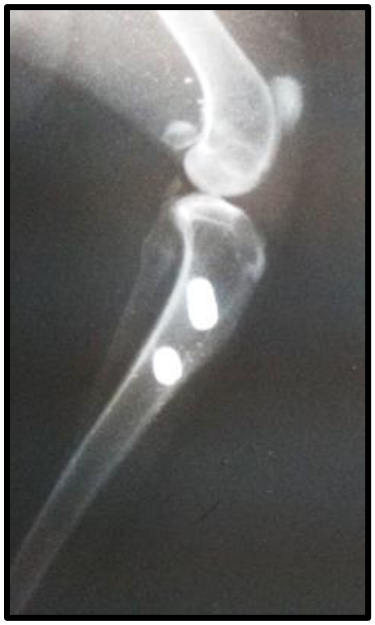


**Figure 2.39: Right tibia after 6 weeks.**

## 2.2.7 Mechanical testing (Removal torque test)

### 2.2.7.1 Materials and equipment

Sterile surgical tools and anesthetic solutions, similar to those used during the implantation process, were used in mechanical testing.

### 2.2.7.2 Methods

The (16) rabbits that were categorized for the mechanical test were anesthetized with the same type and dose of anesthetic solution that was used in the implantation surgical procedure. Then incisions were made to the skin and the muscles at the lateral side of the tibia followed by the flap reflection to expose both implants**.**

The stability of each exposed implant was checked by using handle ends of two instruments. Later, the head of the torque meter screw driver was located inside the slit of the implant head. With continually firm supported bone, to prevent any incorrect measurement, the highest torque value necessary to loosen the implant was recorded, the removal torque value was measured in Newton. Centimeter (N.cm), (**Figure 2.40**). Finally, the surgical flap was sutured and

post-operative care was provided by using the same procedure performed with the implantation processes.

**Figure 2.40: Digital torque meter reading.**

## 2.7.4 Histological testing

### 2.7.4.1 Materials and equipment

Using sterile surgical tools and anesthesia similar to that used during the implantation process. While the additional material and equipment were include:

1-Disc and mandrel.

2- Canada balsam (Batch NO. 10862501, European Union).

3- Distilled water.

4- Ethanol alcohol 96%.

5- Formaldehyde 37%.

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

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

8- Prosthetic engine (Marathon Saeyang Microtech, Korea).

9- Optical microscope (Olympus /542037, Japan).

10- Microscopic glass slides and covers (China).

11- Paraffin wax (Hinweis, U.K.D).

**2.7.4.2 Method**

From both 2nd and 6th weeks healing periods 2 rabbits were anesthetized by overdose anesthetic solution and then scarified for histological examination by optical microscope. A skin incision was made to reveal the screws, then a disc with slow speed handpiece rotation and cooling saline was used for bone tissue cutting around the screws (**Figure 2.41)**. Cutting was performed 5 mm away from the screws in preparation of a bone implant block for histological study **(Figure 2.42).**

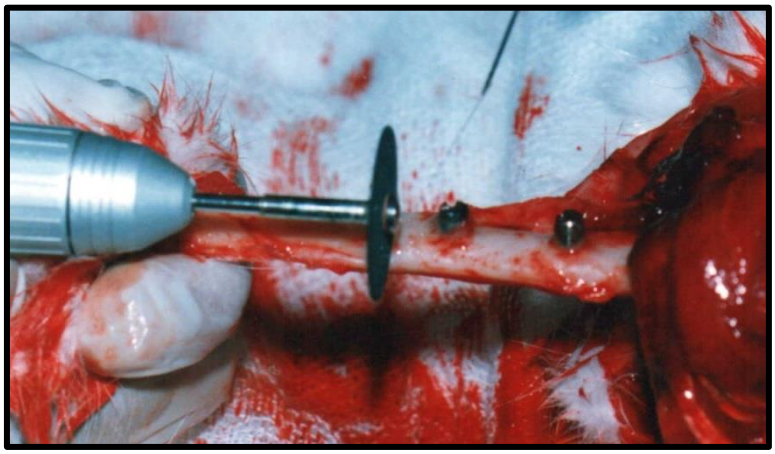


**Figure 2.41: Cutting of bone to prepare bone- implant block.**

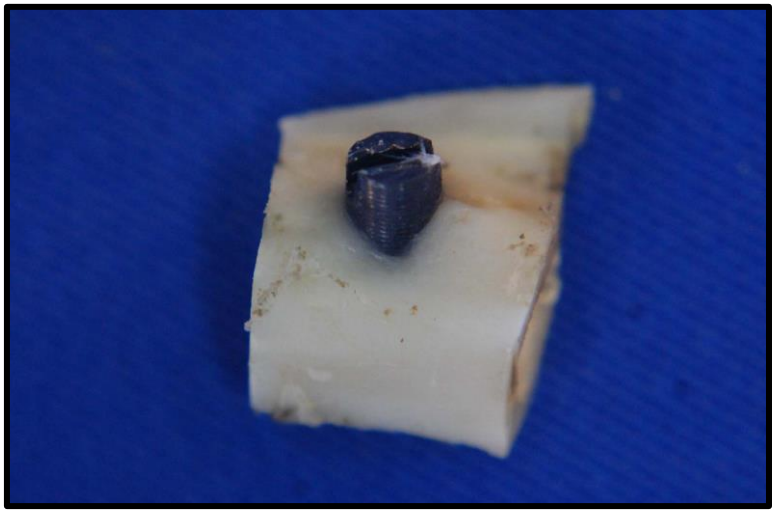


**Figure 2.42: Bone implant block.**

Bone-implant blocks were immediately kept in 10 % fresh formalin solution for 3 days for fixation (**Figure 2.43).** When fixation was completed a series of laboratory procedures were carried out.


**Figure 2.43: 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 placed in the center of melted paraffin dish 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 5 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.

**2.7.4.3 New bone measurement**

Examination of the slides were done by using light microscope with a magnification power 4x all images were taken by I phone 7 camera by using the labcam adapter for iphone 7\8 as shown in **(Figure 2.44)**


**Figure 2.44: Lab cam adapter.**

The new bone formation was measured for each section by using a computer program (Image J version 1.52) (**Figure 2.45)**, by measuring the average diameter of microscope and image then insertion in the set scale box of the program after that the new bone formation percentage was measured for 2weeks and 6 weeks interval according to the following formula: (**Ott, 2008, Baek, *et al.*, 2011).**

$$\text{NBFP\%} = \frac{\textbf{Area of new formed bone}}{\textbf{Total tissue area}} \times 100$$

The mean, standard devasion, standard error for the NBFP were calculated and analyzed by t-test.

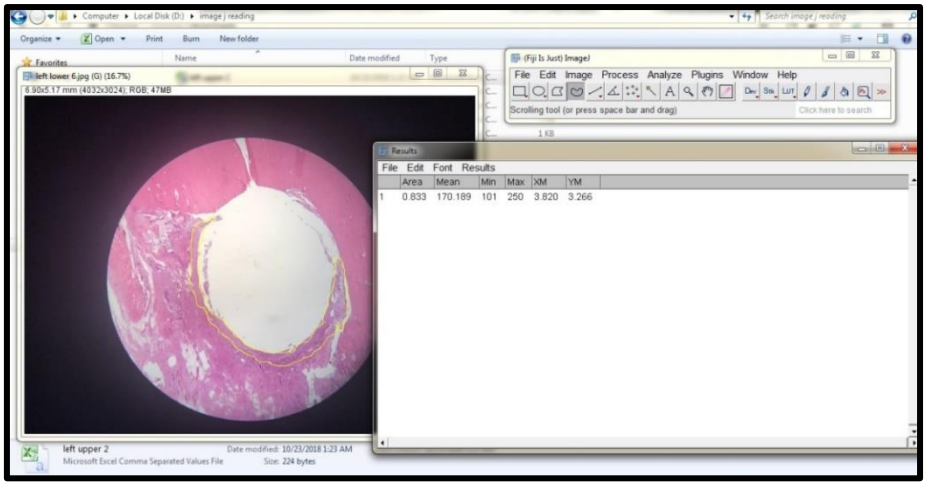



**Figure 2.45 Screenshot of image j.**

## 2.7.5 Statistical analysis

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

**I-Descriptive statistics**

1. Statistical tables.

2. Summary statistic of the readings distribution (mean, SD, SE, minimum & maximum).

3. Bar –chart for graphical presentation.

**II- Inferential statistics**

These were used to accept or reject the statistical hypotheses by testing the quality of means through analysis of variance (ANOVA). Levens test for homogenty, (Tukey HSD test) and (Games-Howell test) 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

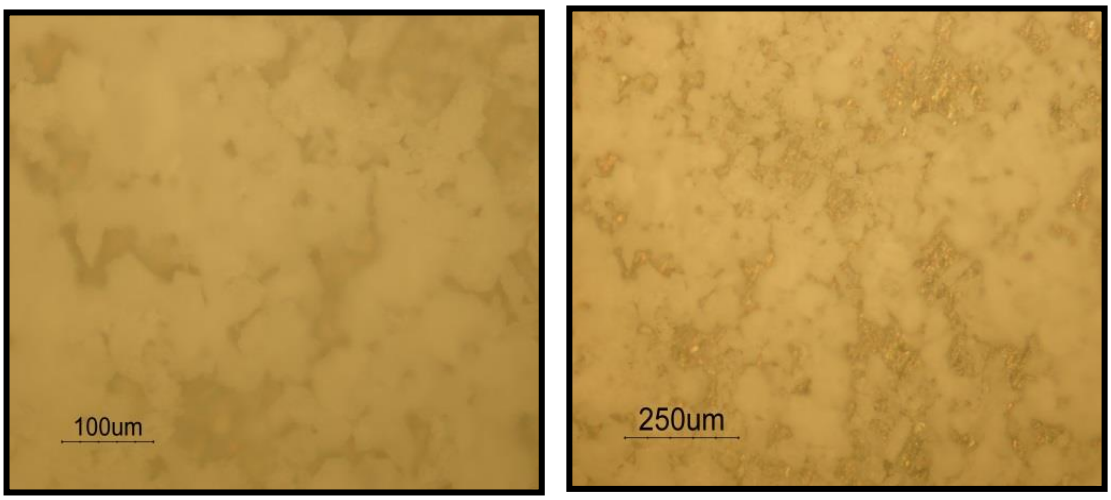
# Results

# Results

## 3.1 In vitro experiments:

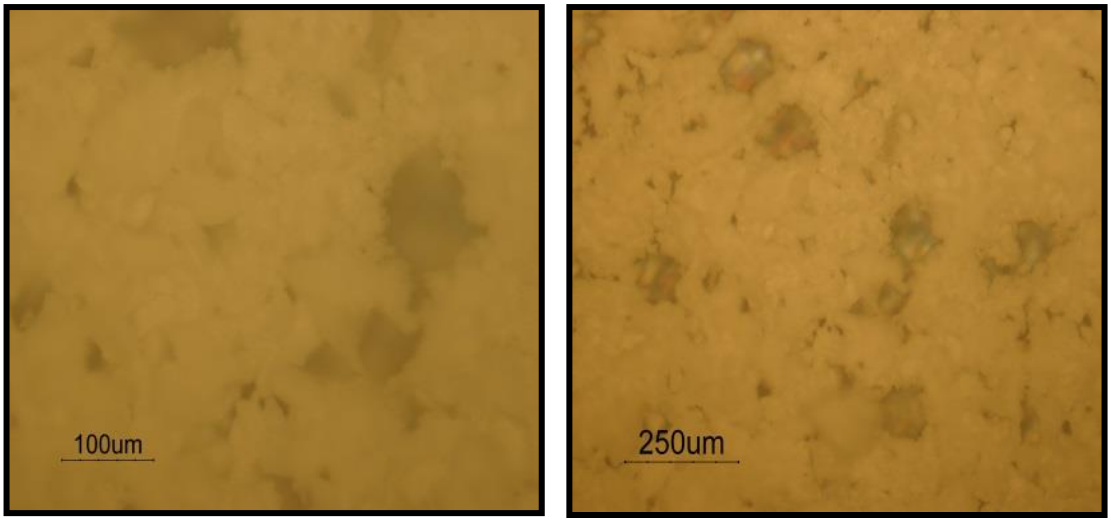
### 3.1.1 Optical microscopical observation

A numbers of micrograph explain the microstructure of nano calcium sulfate using Polyvinyl alcohol (PVA) as binding agent coated the Cp Ti (**Figure 3.1),** nano calcium sulfate using (PVA+PVP polyvinylpyrrolidone) as binding agent coated Cp Ti (**Figure 3.2)** for the same time under different magnification power (100µm, 250µm).




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

**Figure 3.1: Nano calcium sulfate coating with Polyvinyl alcohol A(100µm) B( 250µm).**




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

**Figure 3.2: Nano calcium coating with (Polyvinyl alcohol + Polyvinylpyrrolidine) A(100µm) (250µm).**

## 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 was increased with the increasing of the coating time **(Figure 3.3).**

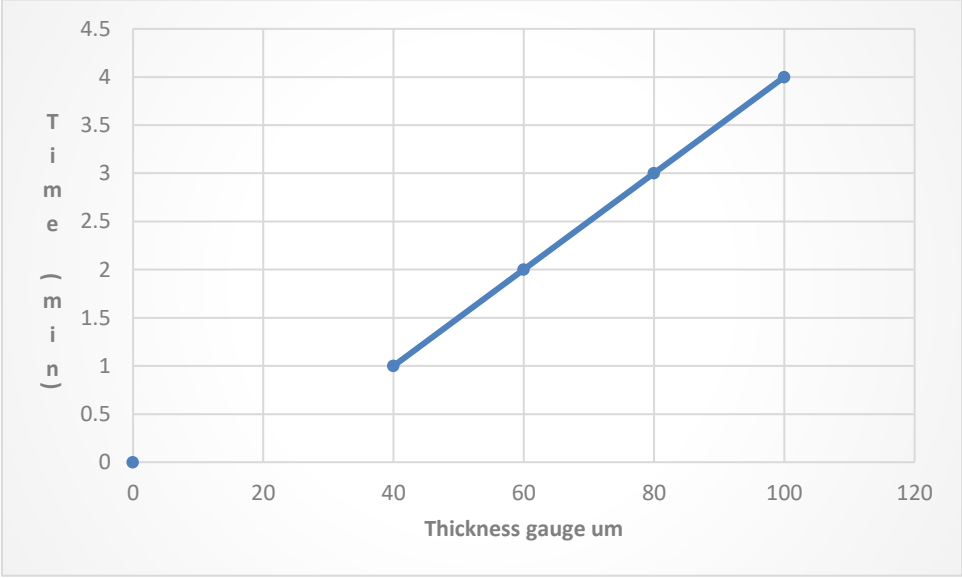



**Figure 3.3: The relation of nano calcium sulfate coating thickness layer with deposition time.**

### 3.1.3 Atomic force microscope observations

The surface roughness analysis produced by the atomic force microscope for Cp titanium, coated by nano calcium sulfate using PVA as binding agent and another sample that use PVA and PVP as binding agents. The first sample show average roughness of 2.53 nm and the granulation distribution chart of the film produced by using this solution is shown in **(Figure 3.4).**

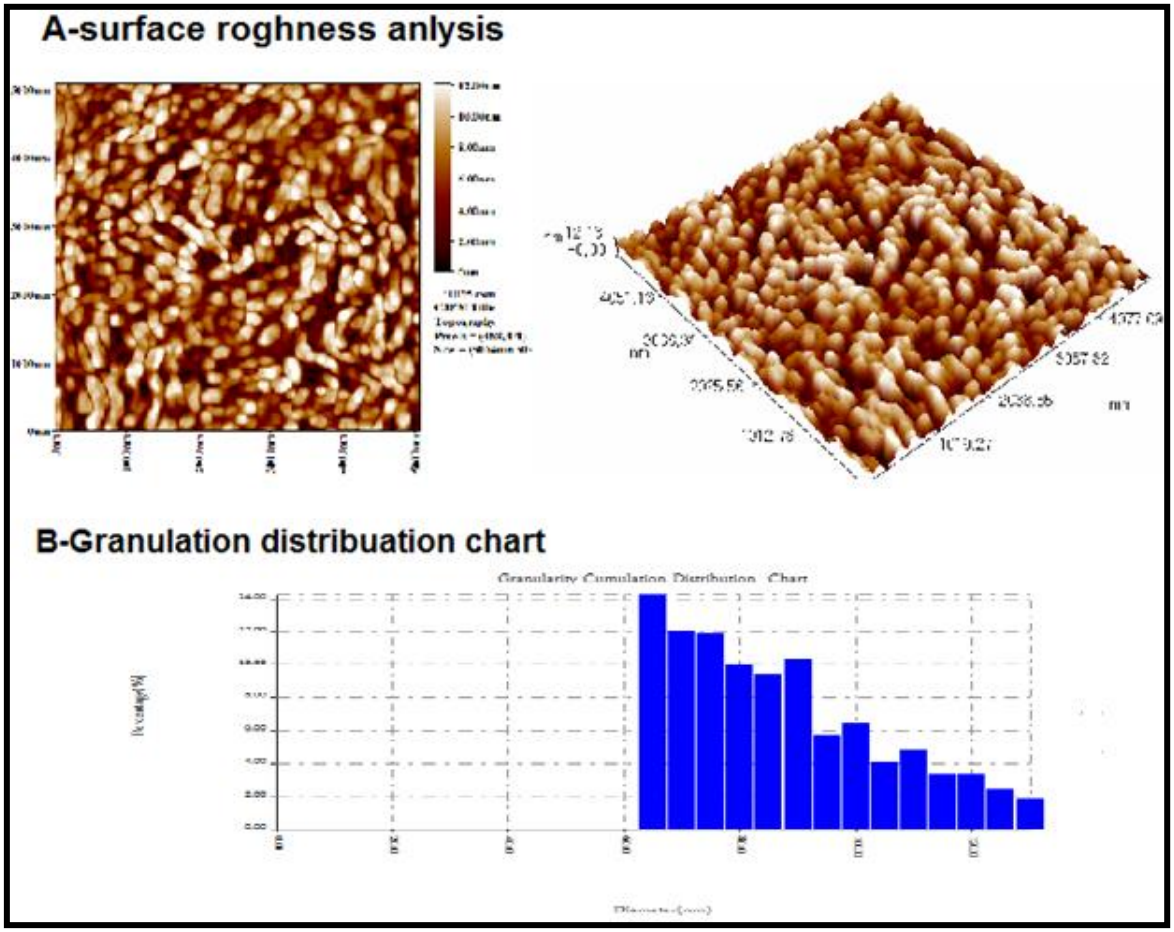



**Figure 3.4 A-Surface roughness analysis and B- Granulation distribution chart for Cp Ti sample coated by using Nano calcium sulfate and PVA as binding agent.**

The second sample where PVA and PVP are used as a binding agent show average roughness 3.28 nm and the granulation distribution chart of the film produced by using this solution is shown in **(Figure 3.5).**

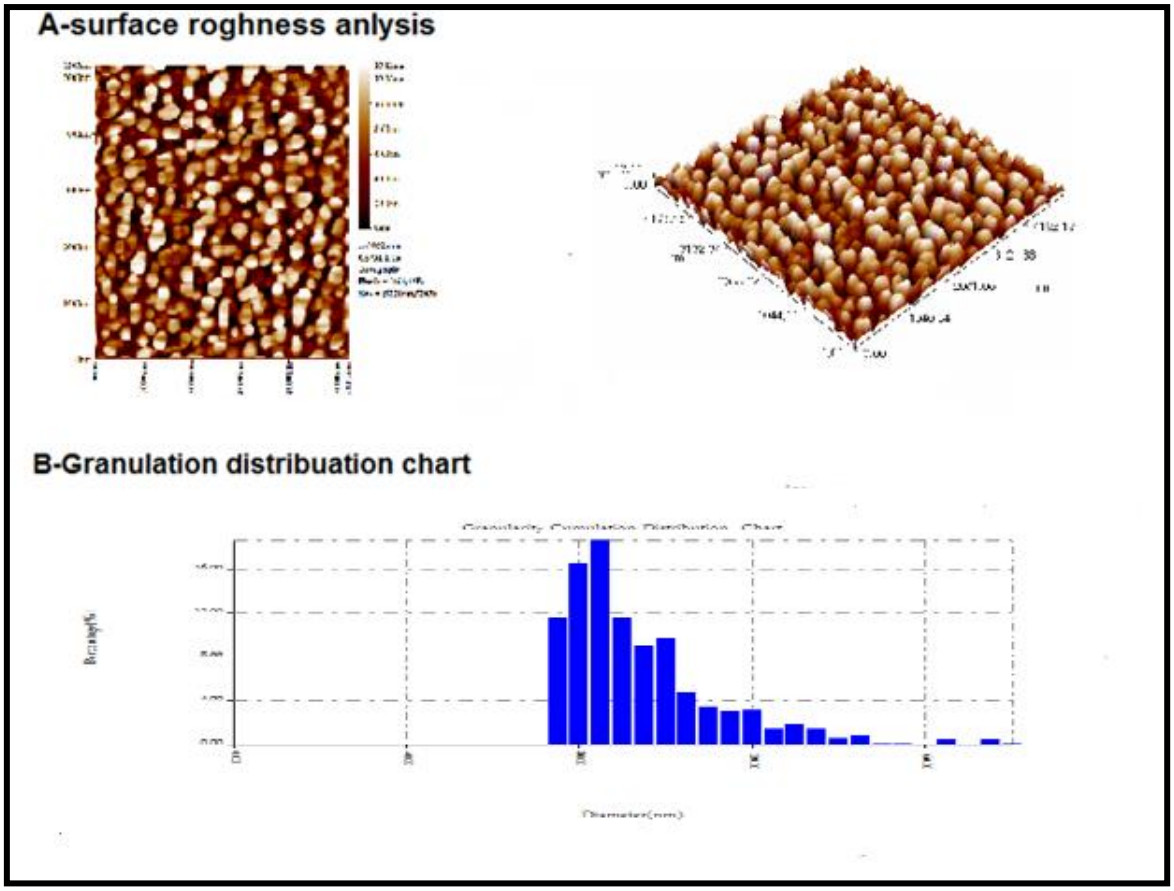



**Figure 3.5 A-Surface roughness analysis , B- Granulation distribuation chart for Cp Ti sample coated by Nano calcium sulfate and (PVA and PVP) as binding agent.**

From the result of optical microscope and atomic force microscope, the nano calcium sulfate solution appear to be the most effective when used with (PVA, PVP) as binding agent when compared to nano calcium sulfate solution that use only PVA as binding agent. The coating film properties produced by this solution were most uniform, less porous, with minimum lost parts of the coating, without noticeable cracks, with good average roughness and the best granulation distribution.

\

## 3.1.4 Phase identification

The XRD pattern for titanium coated with calcium sulfate nano powder with presence of PVA in (**Figure 3.6**) shows an identical match with (ICDD 37-1496) for calcium sulfate in peaks (111), (020), (121), (040) and (224) and identical match with (ICDD 44-1294) for titanium in peaks (002), (101) and (102).The PVA doesn't appear because it is evaporated after heat treatment.

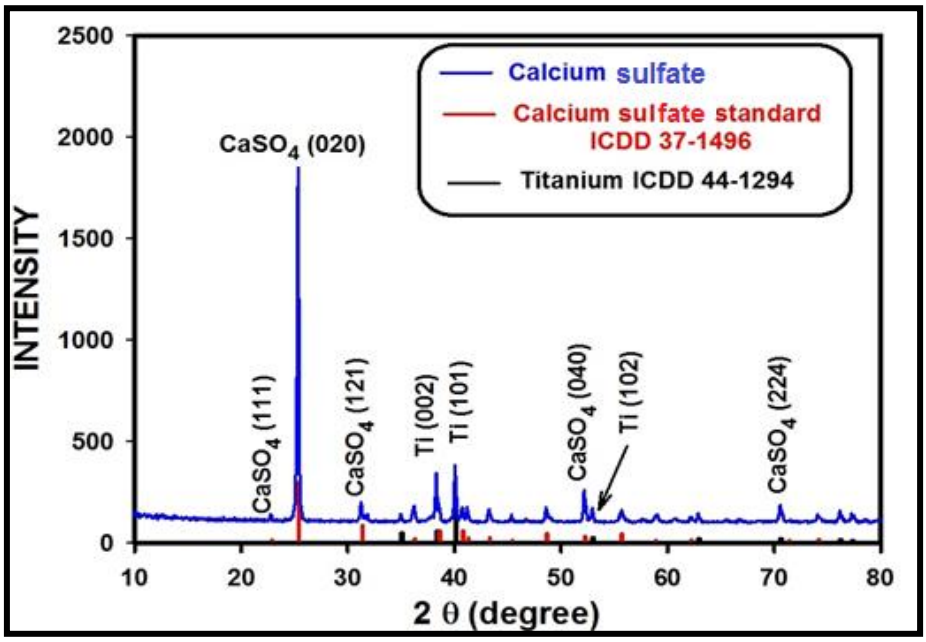



**Figure 3.6 X-ray diffraction pattern of Cp Ti coated with nano calcium sulafate and PVA as binding agent.**

The XRD pattern for titanium coated with calcium sulfate nano powder with presence of PVA and PVP in (**Figure 3.7)** shows an identical match with (ICDD 37-1496) for calcium sulfate in peaks (111) , (020), (121), (040) are (224), and identical match with (ICDD 44-1294) for titanium in peaks (100), (002), (101) and (102).The PVA and PVP didn't appear because they are evaporated after heat treatment .

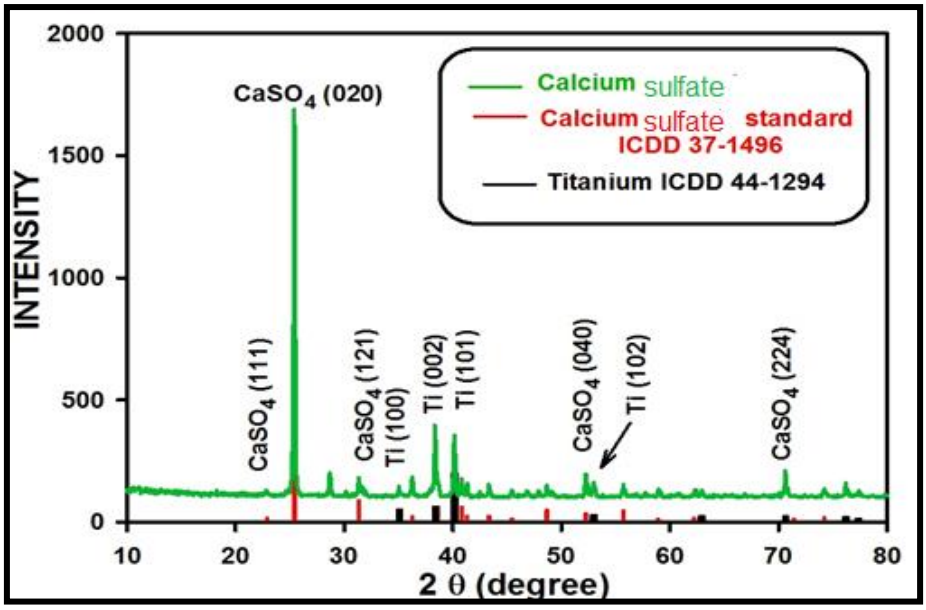



**Figure 3.7 X-ray diffraction pattern of Cp Ti sample coated with nano calcium sulfate and (PVA, PVP) as binding agent.**

The XRD pattern for egg shell with particle size less than 50 µm in **(Figure 3.8)** shows identical match with (ICDD 29-0305 ) in peaks (002), (112), (202), (112), (013) and (032), and there was a little shift in peaks ((302),(204) and (821).

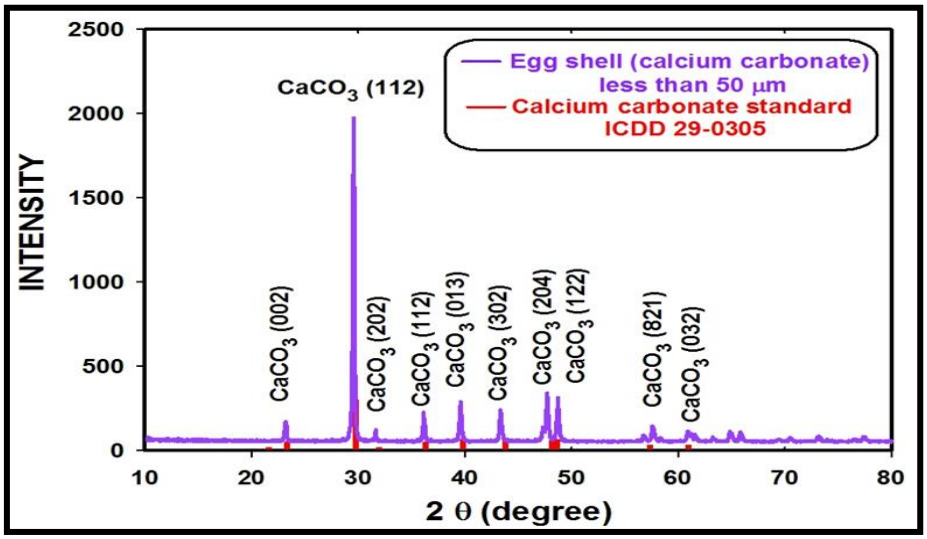



**Figure 3.8 X-ray diffraction patterns for egg shell with particle size less than 50 µm.**

The XRD pattern for egg shell with particle size of 150 µm in **(Figure 3.9)** shows identical match with (ICDD 29-0305 ) in peaks (002), (112), (202),

(112), (013), (032) and (821) and there was a little shift in peaks (302) and (204), this was different from the result in **(Figure 3.8)**

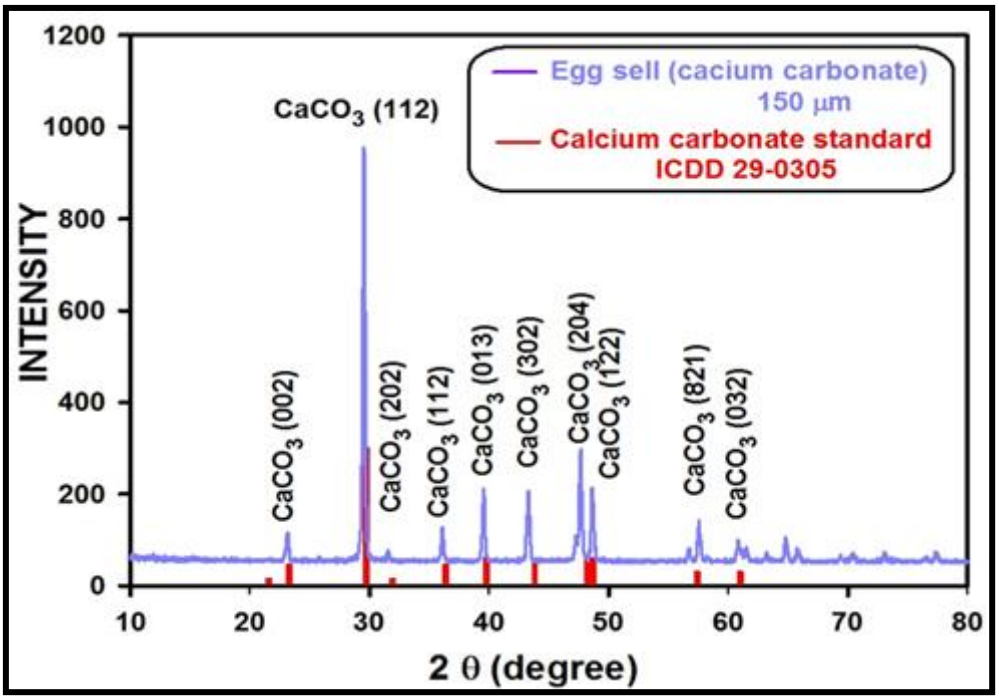



**Figure 3.9 X-ray diffraction pattern for eggshell particle size of 150 µm.**

### 3.1.5 Microhardness test

An average of four readings from four CpTi samples, the first group contain uncoated samples or specimens, the second group contain specimen that coated by 2 layer each one (60 sec), the third group specimen that coated by 3 layer of each one (60sec) and the fourth group contain specimen coated with 4$^{th}$ layer each one of (60 sec). The Vickers microhardness tester were taken to describe the indentation hardness, Vickers hardness was installed to use (1) Kg load and (5) sec loading time, the average hardness Vickers (HV) numbers are seen in **Table 3.1.**

**Table 3.1: The result of microhardness test in Vickers numbers(HV).**

| Group name | Average hardness |
|---|---|
| **Uncoated sample** | 230.4 |
| **Two layer coated sample** | 237.6 |
| **Three layer coated sample** | 248.6 |
| **Four layer coted sample** | 254.3 |

### 3.1.6 Nano-morphological surface analysis

Morphological analysis, by using SEM for Cp Ti discs (uncoated and coated with nano calcium sulfate by using of dip coating techniques), are shown in **(Figure 3.10 and Figure 3.11).**

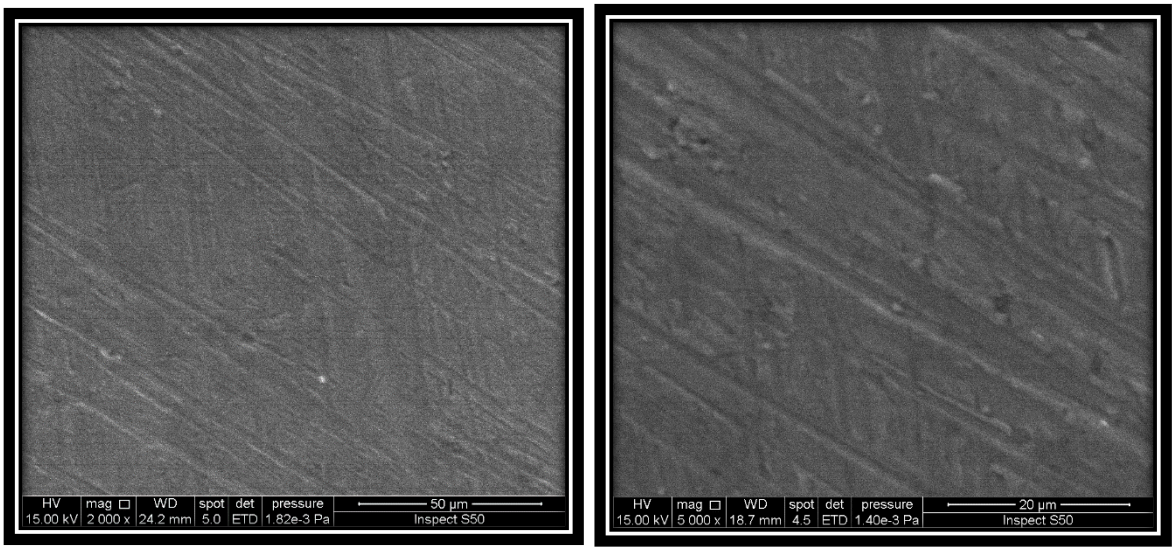
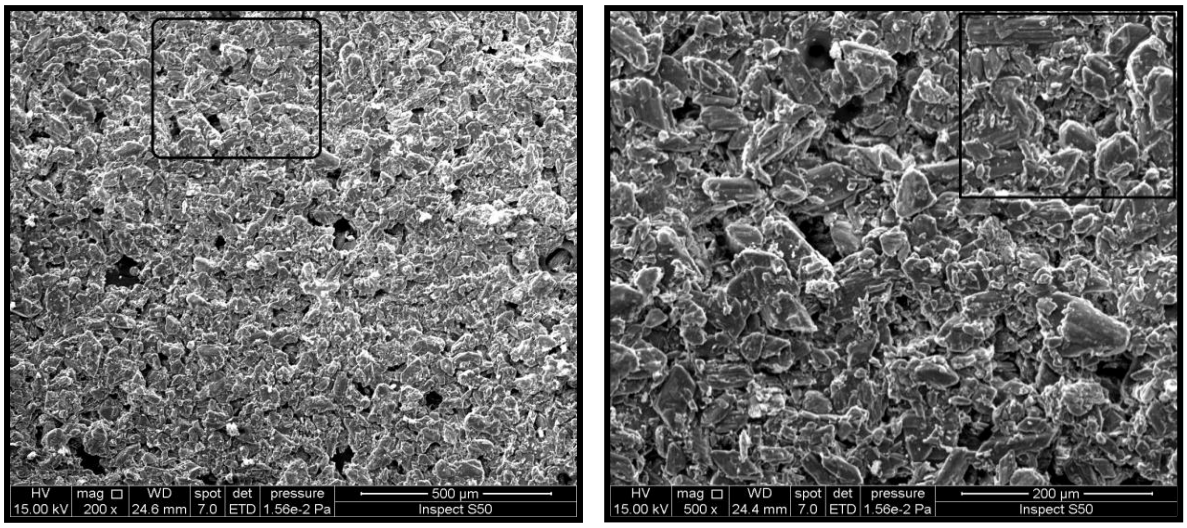
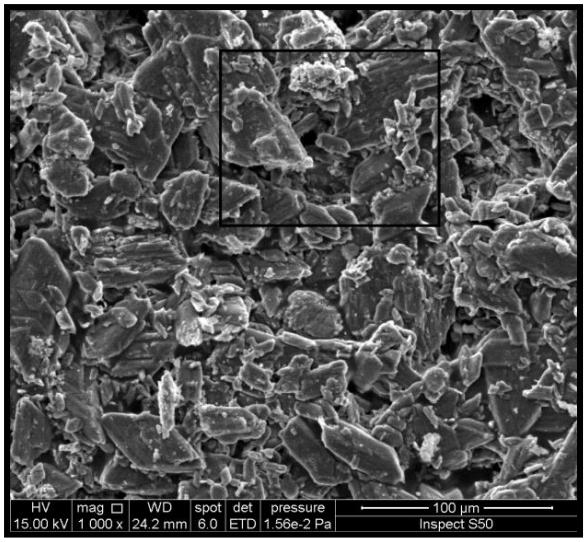


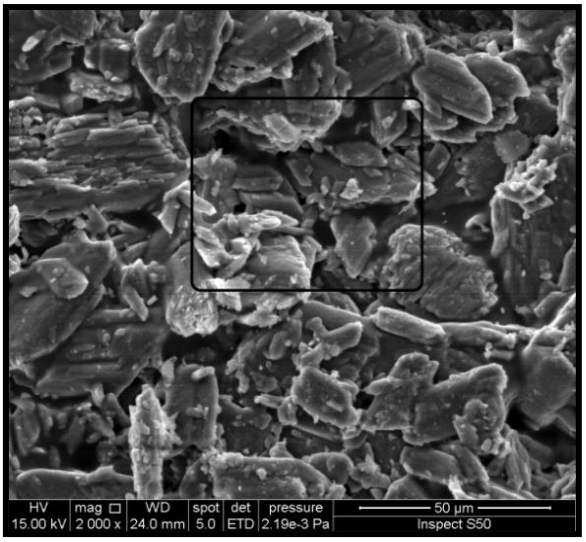

**Figure 3.10 SEM of uncoated Cp Ti disc with different magnifications power (50, 20 µm.)**



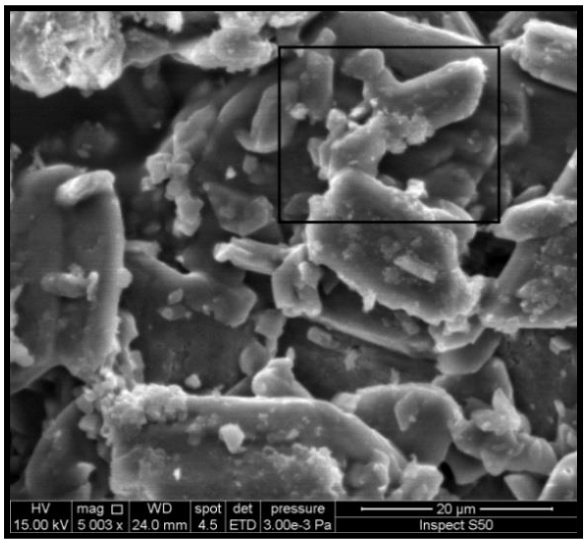

**A (500 µm)** **B(200 µm)**



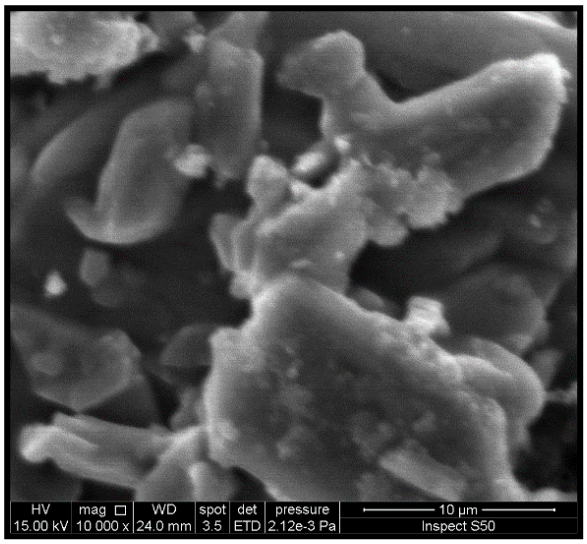

**C (100 µm)**



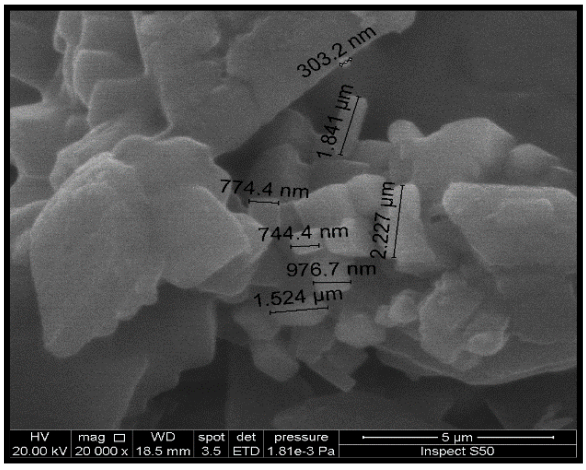

**D (50 µm)**



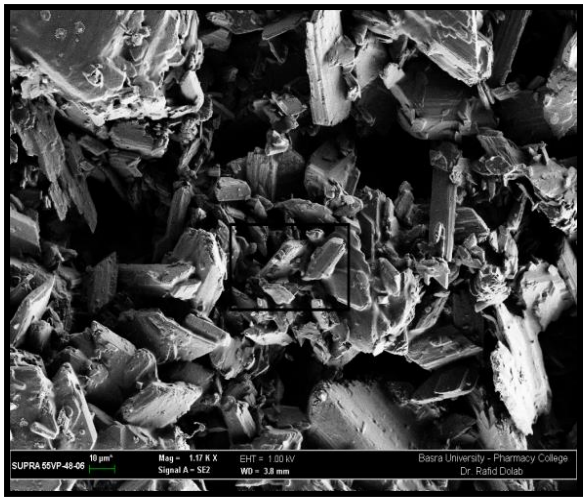

**E (20 µm)**




**F (10 µm)**

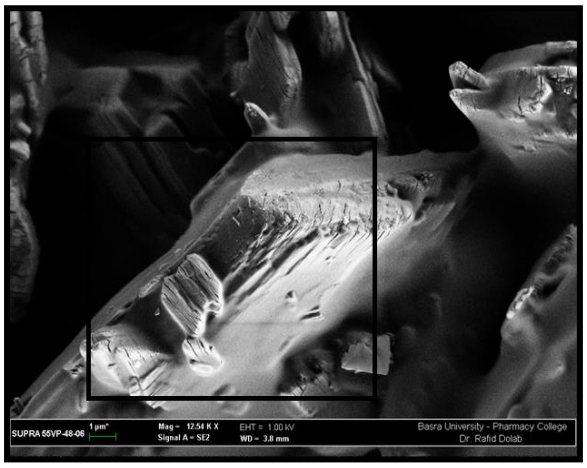



**G (5 µm)**

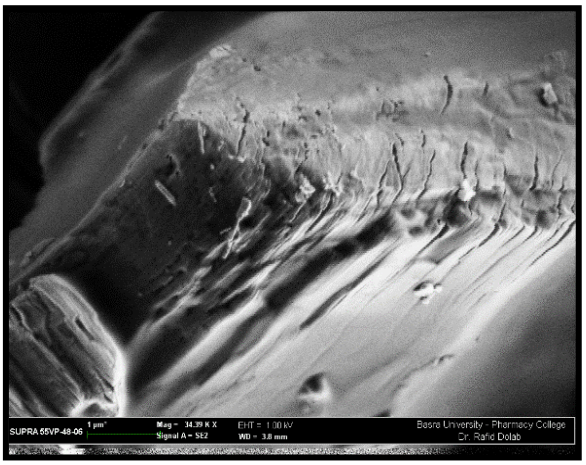



**J (10 μm)**


**K (2 μm)**




**I (1 μm)**




**O (1 μm)**

**Figure 3.11 SEM of coated Cp Ti samples by dip coating techniques with several magnifications (A500,B200,C100,D50,E20,F10,G5,I2,O1) μm.**

## 3.1.7 Elemental composition

Energy dispersive X-ray spectroscopy (EDX) analysis, for the main components of Cp titanium discs (uncoated, nano calcium sulfate disc by using of dip coating technique) including weight and atomic percentages of the main elements, are shown in **(Table 3.1 and Table 3.2)**.

**Table (3.2): EDX analysis for uncoated commercial pure titanium disc.**

| Element | Weight % | Atomic % |
|---|---|---|
| **Ti K-series** | 96.98 | 91.47 |
| **O K-series** | 3.02 | 8.53 |
| **Total** | 100.00 | 100 |

**Table (3.3): EDX analysis for nano calcium sulfate coated Cp Ti discs by using dip coating technique.**

| Elements | Weight% | Atomic% |
|---|---|---|
| **O K-series** | 53.78 | 60.58 |
| **C K-series** | 16.31 | 24.39 |
| **Ca K-series** | 15.84 | 7.10 |
| **S K-series** | 11.89 | 6.66 |
| **Cl K-series** | 1.07 | 0.04 |
| **Na K-series** | 0.80 | 0.63 |
| **Al K-series** | 0.03 | 0.02 |
| **Si K-series** | 0.08 | 0.05 |
| **P K-series** | 0.04 | 0.02 |
| **Total** | 100.00 | 100.00 |

An EDX series usually displays peaks corresponding to the energy levels of which the most X-rays received. Each of these peaks is unique to an atom and therefore corresponds to a single element. The higher the peak in a spectrum, the more concentrated the element is in the specimen **(Anish, *et al.*, 2013).** EDX plots of Cp Ti discs (uncoated and nano calcium sulfate coated discs by using of dip coating technique), which obtained by accelerating voltage of 3 kV, are shown in (**Figure 3.11 and Figure 3.12).**

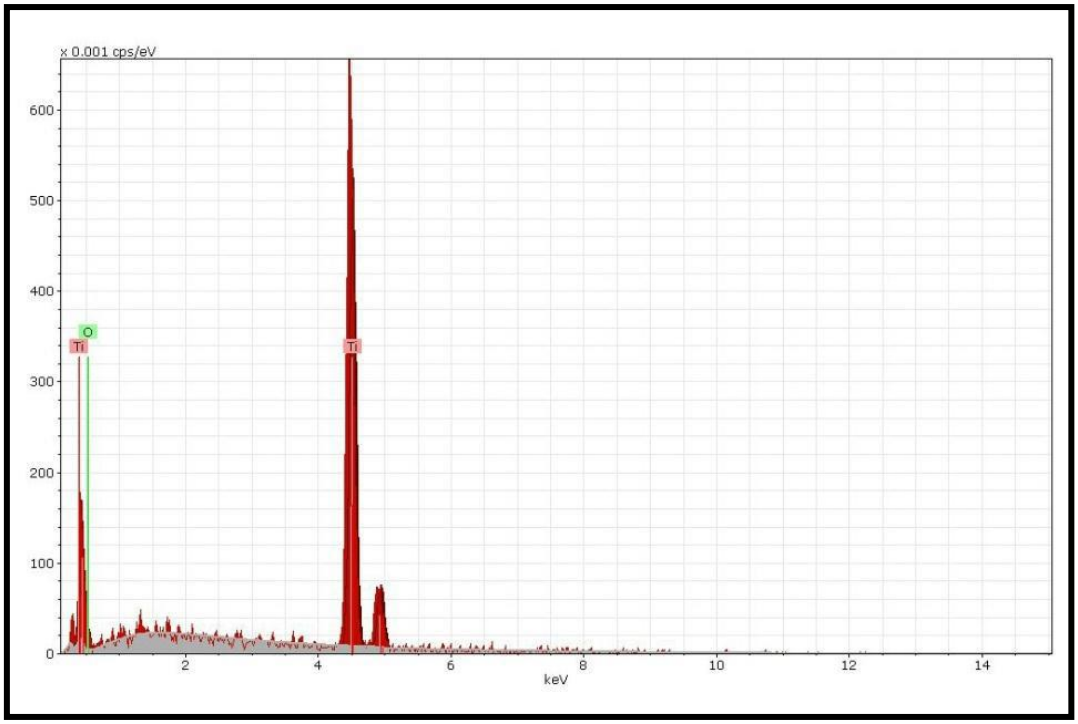



**Figure 3.11 EDX analysis for uncoated disc.**

EDX analysis showed the presence of Oxygen, Carbon and Calcium as the major component in coated Disc. SEM\EDX mapping showed Titanium even distribution in dip coated disc (**Figure 3.12 and Figure 3.13).**

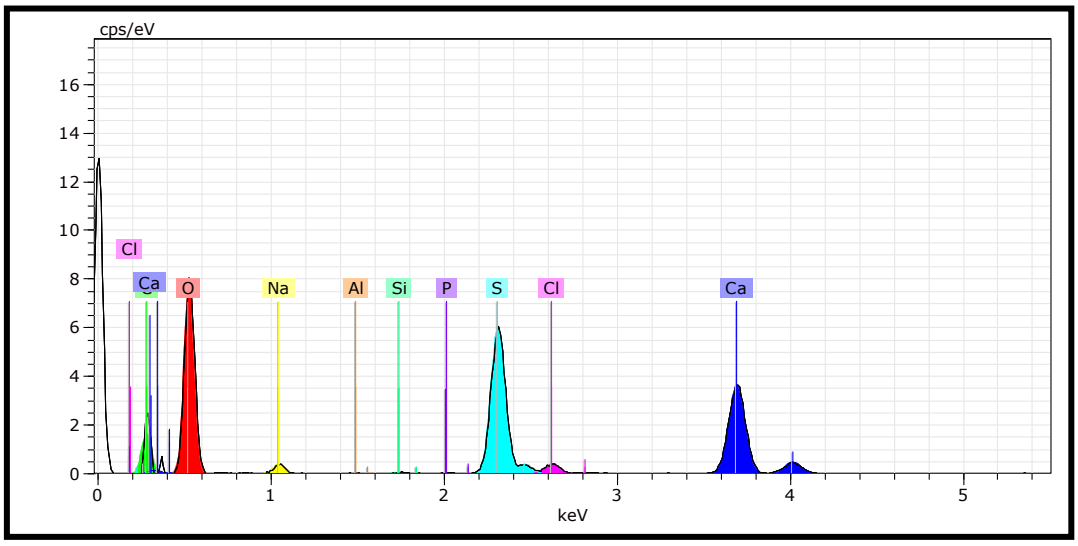



**Figure 3.12: EDX analysis for nano calcium sulfate coated disc.**

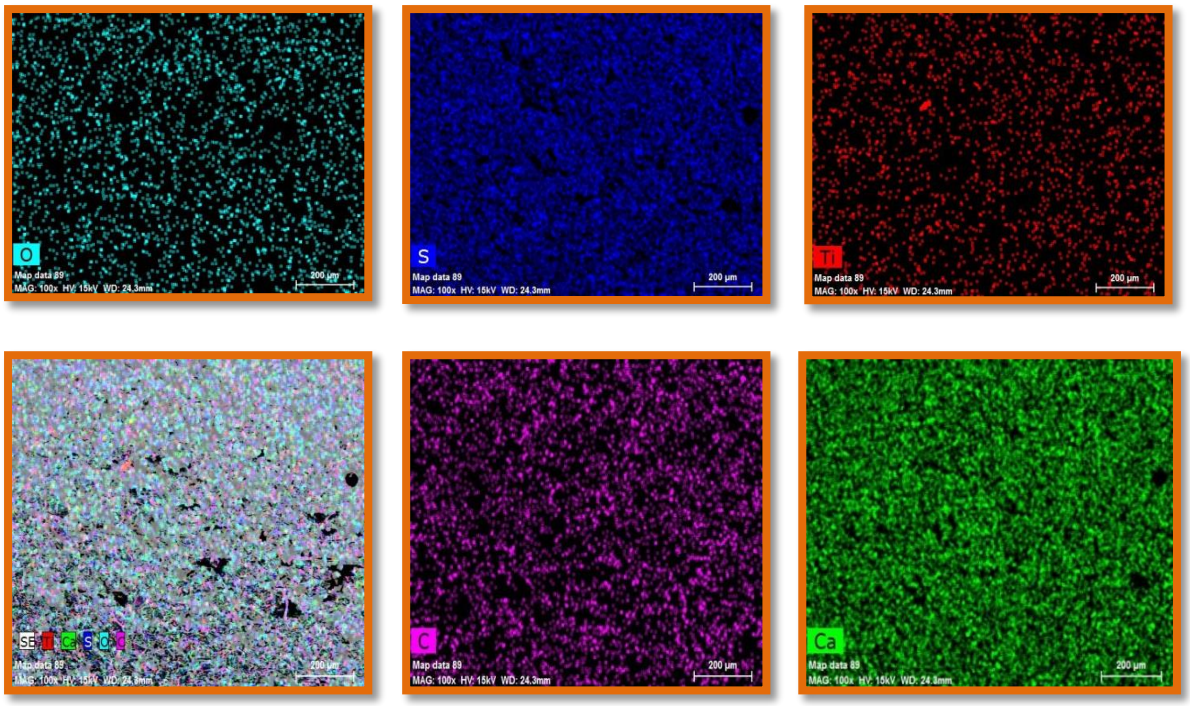



**Figure 3.13: EDX mapping for nano calcium sulfate coated disc.**

### 3.1.8 FTIR analysis for eggshell powder

Spectra for eggshell powder with particle size less than 50µm and egg shell powder with particle size of 150µm. Spectra of eggshell powder with particle size less than 50µm showed peak 1433 heavily related with carbonate material inside the matrix of egg shell (**Dean, 1987).** While the peaks 710 and 876 are concerned with deformation from inner side of plane and deformation from outer side of planar ranges, by sequence, in the existence of $CaCo_3$ (**Busca, *et al.*, 2000).** The remaining peaks (3288, 2979, 2873) related to amines and amides (**Busca, *et al.*, 2000).** The 150µm has the same peak variation but slightly bigger as shown in (**Figure 3.14 and Figure 3.15).**

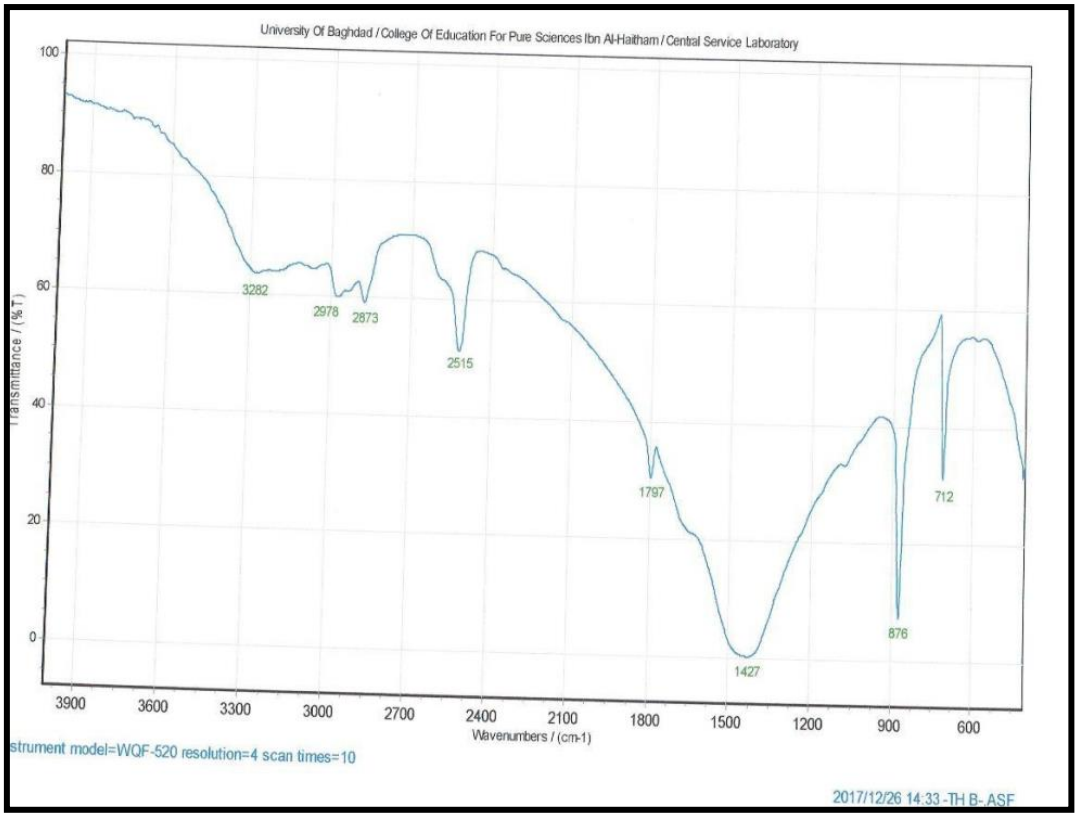



**Figure 3.14: FTIR analysis for eggshell particle of less than 50 µm**

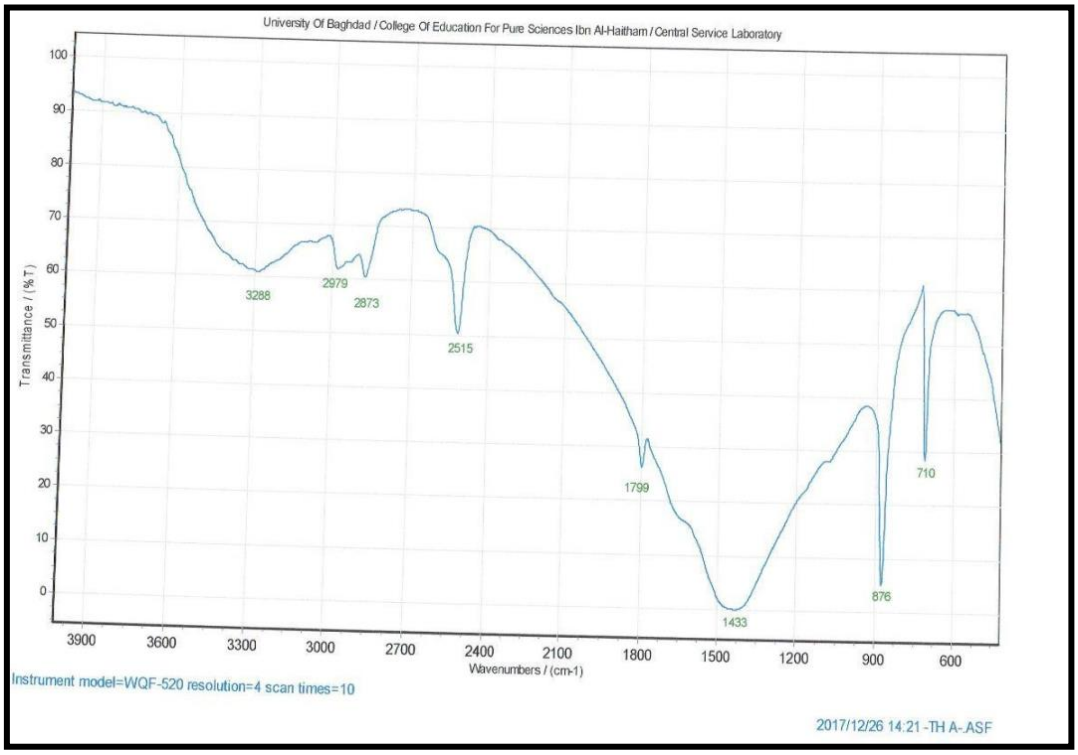



**Figure 3.15: FTIR analysis for eggshell powder of particle size of 150 µm.**

# 3.2 In vivo experiments

## 3.2.1 Clinical observations

All the rabbit recovered well with normal activity at the end of first week of implantation which consider a sign of implant toleration by these rabbit. There is no tissue reaction, sign of gross infection or any negative observation in any rabbit precisely around the implant site at the time that some of rabbit being euthanized. All screws were found stable inside the bone and no movement when manual force applied to it by the end of examination tool. The coronal part of these implants were not diagnosed with detectable peri-implant defects after both two and six weeks healing intervals. The remaining rabbits, after the mechanical (removal torque) test of the implants, were kept in their cages and recovered well after few days of the surgery.

## 3.2.2 Mechanical testing

Descriptive statistics of removal torque mean values of Cp titanium screws, coated with nano calcium sulfate after 2 weeks of implantation, first group (A) was the control group, the second group (B) is just coated with nano calcium sulfate, the third group (C) the screws were coated with nano calcium sulfate and gap made around the implant and filled by eggshell powder, the fourth group (D) the screws were coated with nano calcium sulfate and gap made around the implant and not filled by egg shell powder as shown in **(Table 3.4).**

After two weeks interval, a higher torque mean value was needed to remove the implants coated by nano calcium sulfate and gap made around the implant and filled by eggshell powder (group C) (13.000) N.cm.

**Table 3.4 Descriptive statistical analysis for torque removal test after 2 weeks healing period.**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimm | Maximm |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| **A 2w** | 8 | 8.625 | 1.408 | 0.498 | 7.448 | 9.802 | 7 | 11 |
| **B 2w** | 8 | 10.500 | 1.690 | 0.598 | 9.087 | 11.913 | 8 | 13 |
| **C 2w** | 8 | 13.000 | 1.927 | 0.681 | 11.389 | 14.611 | 10 | 16 |
| **D 2w** | 8 | 9.750 | 1.282 | 0.453 | 8.678 | 10.822 | 8 | 12 |

To know if the sample have equal variances the levene's test is used for 2 week, since the significant value is 0.633 which is more than 0.05, levene's test is non-significant so equal variance is assumed as shown in **(Table 3.5)**, so in this study Tukey HSD multiple compassion was used.

**Table 3.5: Levene's test for torque removal test 2 weeks interval.**

| Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| **580** | **3** | **28** | **0.633** |

The significance difference between groups for 2 week interval were tested by one way ANOVA as shown in (**Table 3.6).** It was found that there is statistically highly significant difference among the experimental groups $P<0.01$.

**Table 3.6: ANOVA for torque removal test of implant screw after 2 week interval.**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| **Between Groups** | 82.594 | 3 | 27.531 | 10.800 | 0.000 HS |
| **Within Groups** | 71.375 | 28 | 2.549 | | |
| **Total** | 153.969 | 31 | | | |

Multiple comparison (Tukey HSD) test in 2 week healing interval revealed that there is highly significant difference between the control group (A) and the third

group (C), at $P< 0.001$ and non-significance between the remaining group at the $P > 0.05$ level as shown in **(Table 3.7).**

**Table 3.7: Tukey HSD comparison test for all groups included in the study for torque removal test for two weeks.**

| (I) Groups | | Mean Difference (I-J) | Sig. |
|---|---|---|---|
| **A 2w** | B 2w | -1.875 | 0.647 |
| | C 2w | -4.375* | 0.003 |
| | D 2w | -1.125 | 0.963 |
| **B 2w** | C 2w | -2.500 | 0.286 |
| | D 2w | 0.750 | 0.997 |
| **C 2w** | D 2w | 3.250 | 0.063 |

Descriptive statistics of removal torque mean values of Cp titanium screws and coated with nano calcium sulfate after 6 weeks of implantation was shown in (**Table 3.8)**, a higher torque mean value was needed to remove the implants coated by nano calcium sulfate and gap made around the implant and filled by eggshell powder (group C) (17.375) N.cm than the remaining group.

**Table 3.8: Descriptive statistical analysis for torque removal test after 6 week healing period.**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| **A 6 w** | 8 | 12.125 | 1.727 | 0.611 | 10.681 | 13.569 | 10 | 15 |
| **B 6w** | 8 | 12.250 | 2.315 | 0.818 | 10.315 | 14.185 | 10 | 17 |
| **C 6w** | 8 | 17.375 | 3.462 | 1.224 | 14.481 | 20.269 | 12 | 23 |
| **D 6w** | 8 | 11.500 | 2.390 | 0.845 | 9.502 | 13.498 | 9 | 16 |

To know if the sample have equal variances the levene's test is used for six week, since the *P* value is 0.4 which is more than 0.05, levene's test is non-significant so equal variance is assumed as shown in **(Table 3.9)** and Tukey HSD test for multiple comparison was used.

**Table 3.9: Levene's test for torque removal test 6 week interval.**

| Levene's Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| **1.016** | **3** | **28** | **0.4** |

The significance difference between groups for 6 week interval were tested by one way ANOVA as shown in (**Table 3.10).** It was found that there is statistically highly significant difference among the experimental groups *P*<0 .01 at three degrees of freedom.

**Table 3.10: ANOVA for torque removal test of implant screw after 6 week interval.**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| **Between Groups** | **178.625** | **3** | **59.542** | **9.148** | **0.000 HS** |
| **Within Groups** | **182.250** | **28** | **6.509** | | |
| **Total** | **360.875** | **31** | | | |

Multiple comparison (Tukey HSD) test in 6 week healing interval revealed that there is highly significant difference between the control group (A) and the third group(C), the second group (B) and third group (C), the third group (C) and fourth group (D) at *P*< 0 .01

While non-significance between the control group (A) and the second group (B), control group (A) and the fourth group (D) and also between the second group (B) and the fourth group (D) at (*P* > 0.05*)*. As shown in (**Table 3.11).**

**Table 3.11: Tukey HSDtest for all groups included in the study for torque removal test for six weeks.**

| (I) Groups | | Mean Difference (I-J) | Sig. |
|---|---|---|---|
| A 6 w | B 6w | -0.125 | 1.000 Ns |
| | C 6w | -5.250* | 0.000 HS |
| | D 6w | 0.625 | 0.999 NS |
| B 6w | C 6w | -5.125* | 0.000 HS |
| | D 6w | 0.750 | 0.997 NS |
| C 6w | D 6w | 5.875* | 0.000 HS |

t – test was performed for testing the equality of means between the two healing interval for all groups presented highly signficance difference at $P<0.01$ in the control group and the second group. Signicance difference at the $P< 0.05$ level presented in the third group (C) and the fourth group (D) as shown in **(Table 3.12).** A bar chart showed the summary of difference in the mean of removal torque value between all groups as shown in **(Figure 3.16)**

**Table 3.12 Paired sample t-test comparison for torque removal test.**

| | | Mean | t | df | Sig. (2-tailed) |
|---|---|---|---|---|---|
| Pair 1 | A_2 - A_6 | -3.500 | -4.365 | 7 | .003 HS |
| Pair 2 | B_2 - B_6 | -1.750 | -4.782 | 7 | .002 HS |
| Pair 3 | C_2 - C_6 | -4.375 | -2.692 | 7 | .03 HS |
| Pair 4 | D_2 - D_6 | -1.750 | -2.824 | 7 | .026 S |

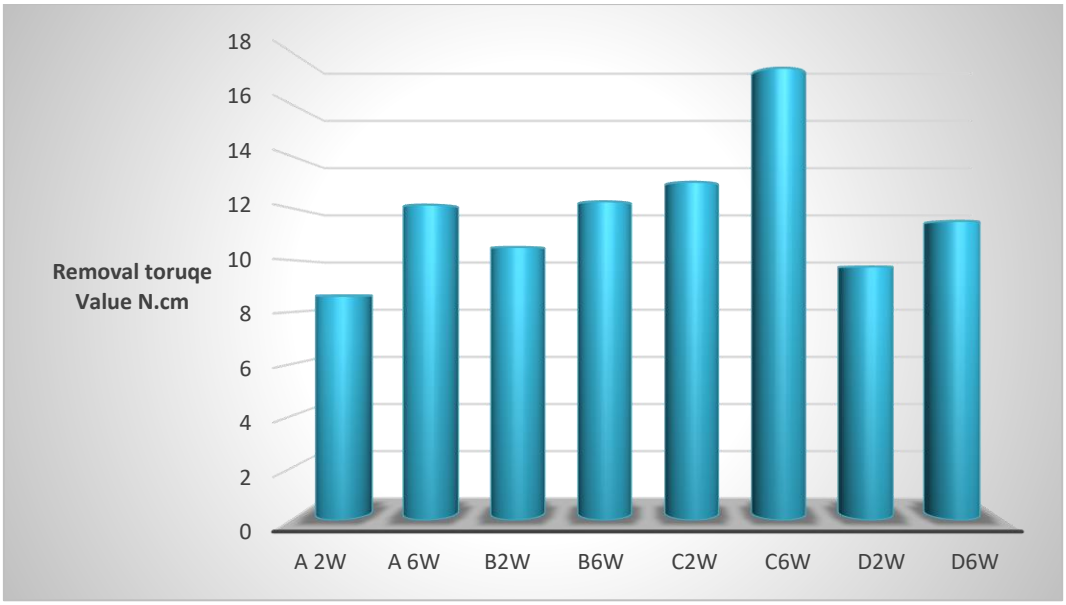



**Figure 3.16: A bar chart showed the summary of the difference in the tourqe mean value between all group.**

### 3.2.3 Histological features after two weeks of implantation

#### 3.2.3.1 Histological features of the uncoated screw group A

After 2 weeks of implantation, the microphotograph at the 40x magnification power showed implant space surrounding by osteoid tissue and at 400x magnification power showed bone trabeculae filled by osteocyte and surrounded by scattered number of osteoblast **(Figure 3.17 and Figure 3.18).**

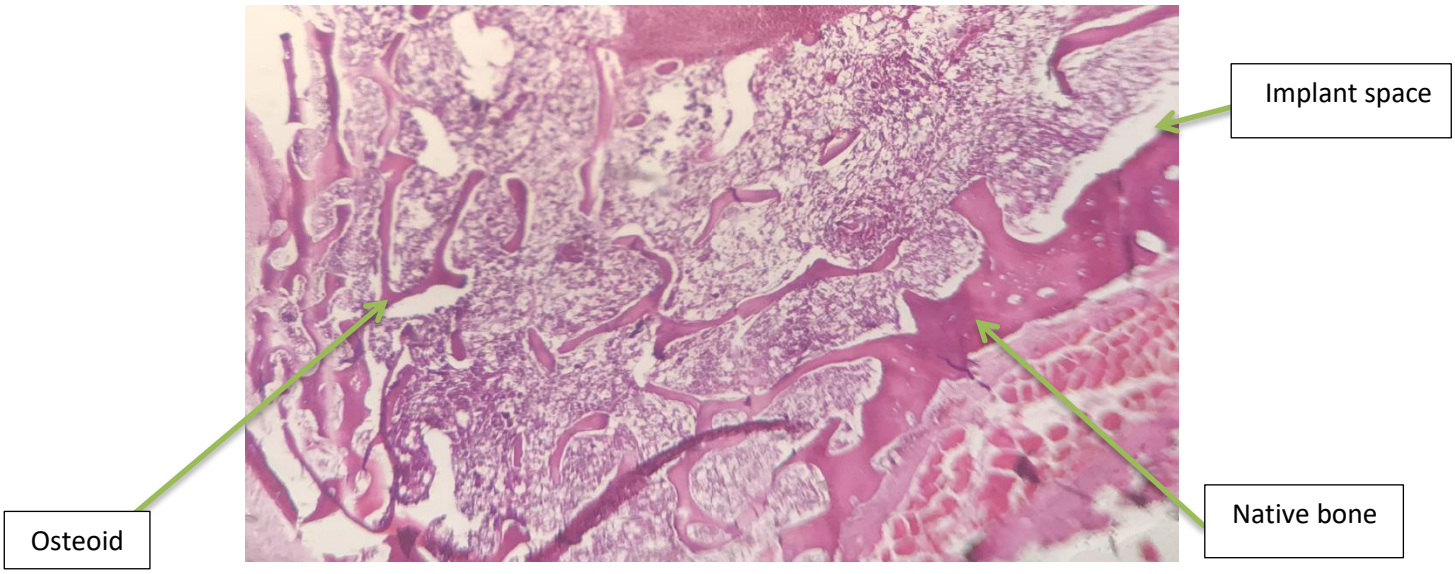



**Figure 3.17: Microphotograph of the control group showed osteoid formation (40x).**

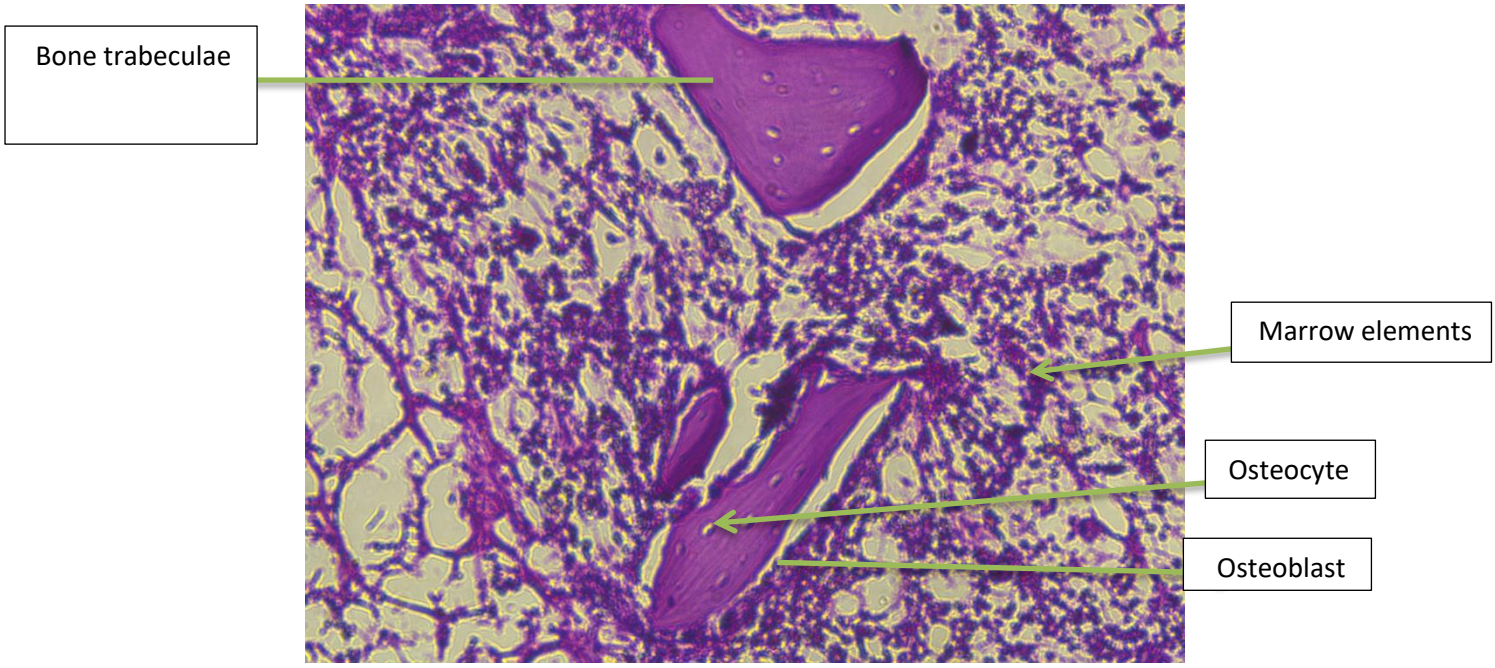



**Figure 3.18: Microphotograph of the control group showed osteoid tissue (400x) after 2 weeks.**

### 3.2.3.2 Histological features of nano calcium sulfate coated screw (Group B) .

After 2 weeks of implantation, the microphotograph at the 40x magnification power showed increased osteoid tissue surrounding the implant space **(Figure 3.19).** The thread area showed new osteocyte **(Figure 3.20)** and surrounded by osteoblasts which is scattered within implant space

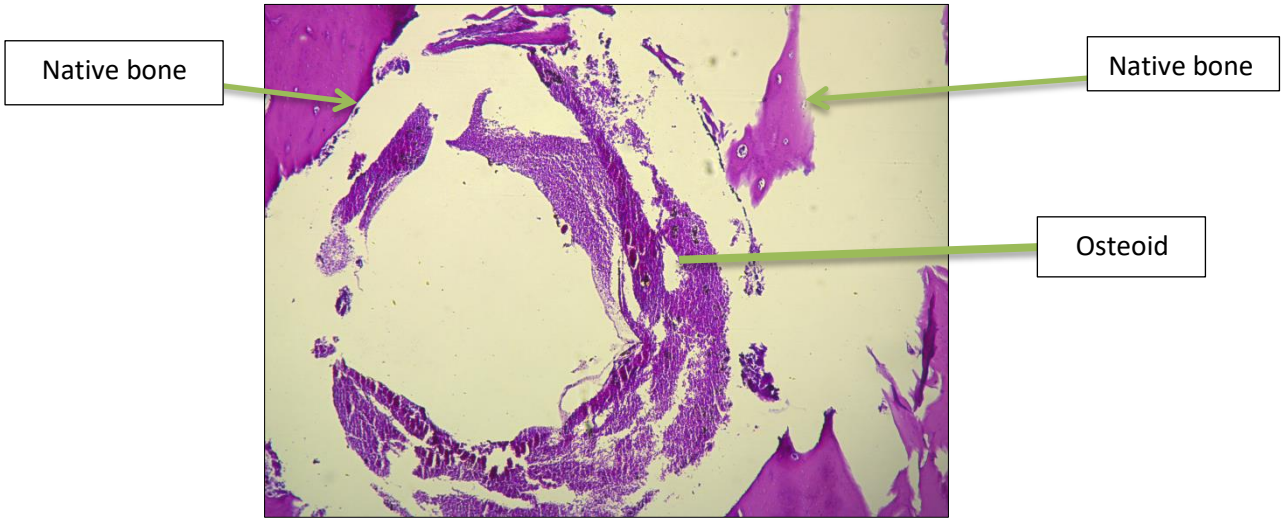



**Figure 3.19: Microphotograph of nano calcium sulfate dip coated group (40x) after 2 weeks.**

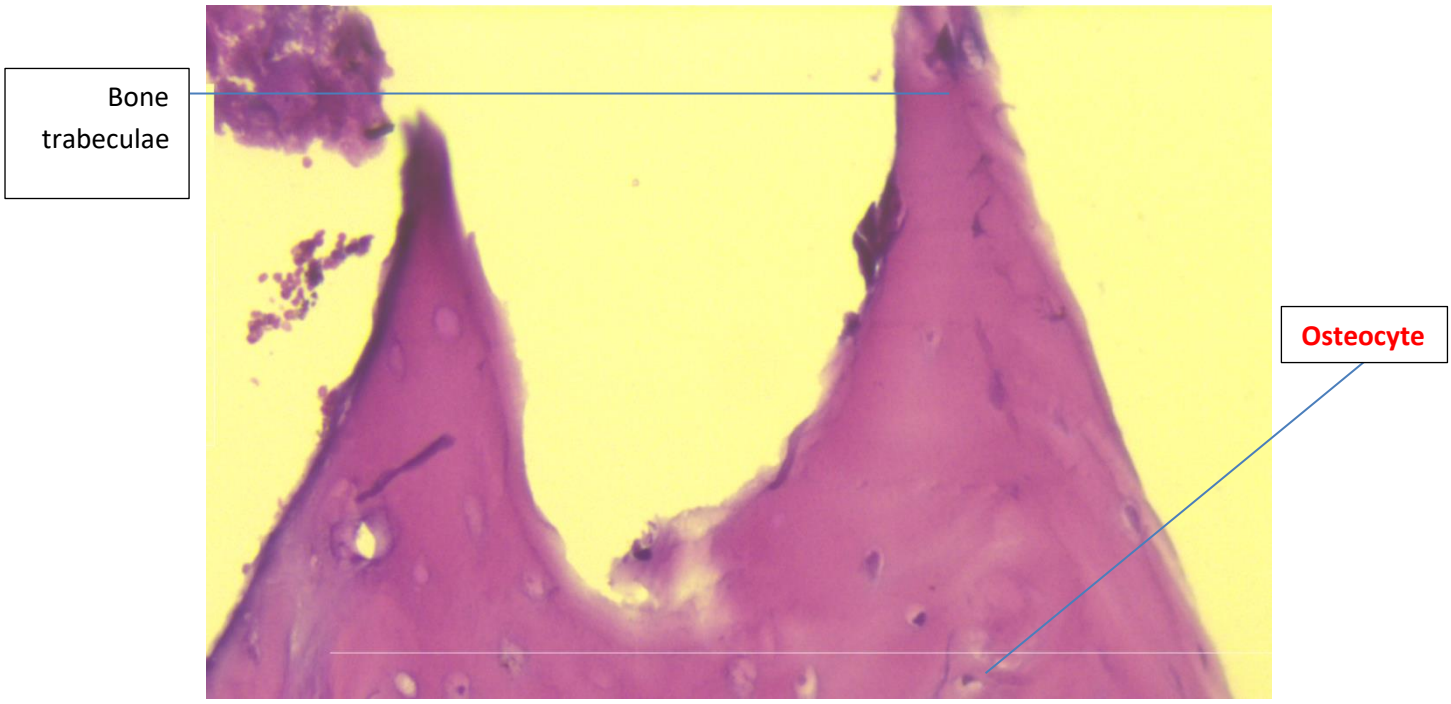



**Figure 3.20 Thread area surrounding the implant space (200x) after 2 weeks**

### 3.2.3.3 Histological feature of nano calcium sulfate coated screw with egg shell powder ( group C).

After 2 weeks of implantation, the microphotograph at the 40x magnification power showed bone trabeculae around the implant space **(Figure 3.21),** which presented with increased numbers of osteocytes (**Figure 3.22)** and more osteoblast scattered within marrow element.

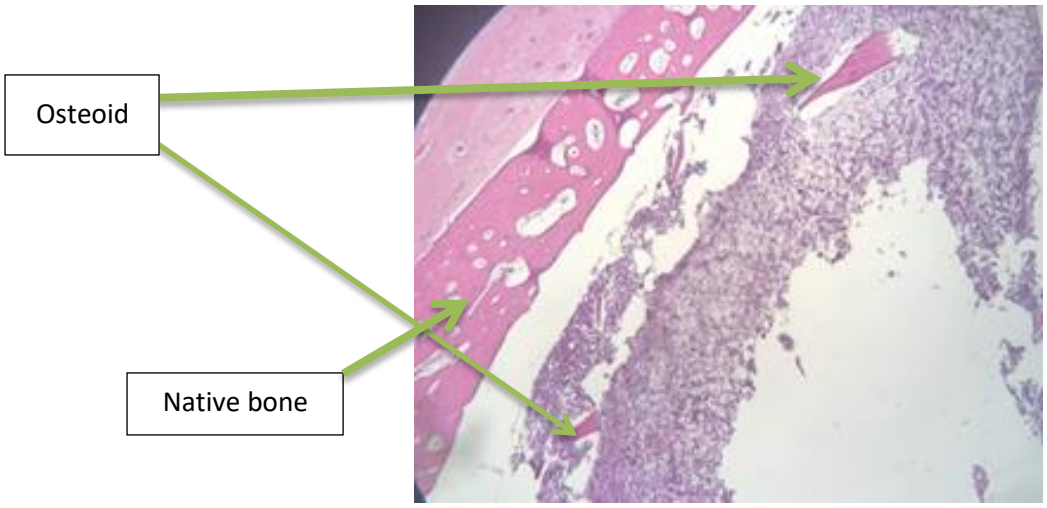



**Figure 3.21 Microphotograph of nano calcium sulfate dip coated with eggshell powder as graft material (40x) after 2 weeks.**

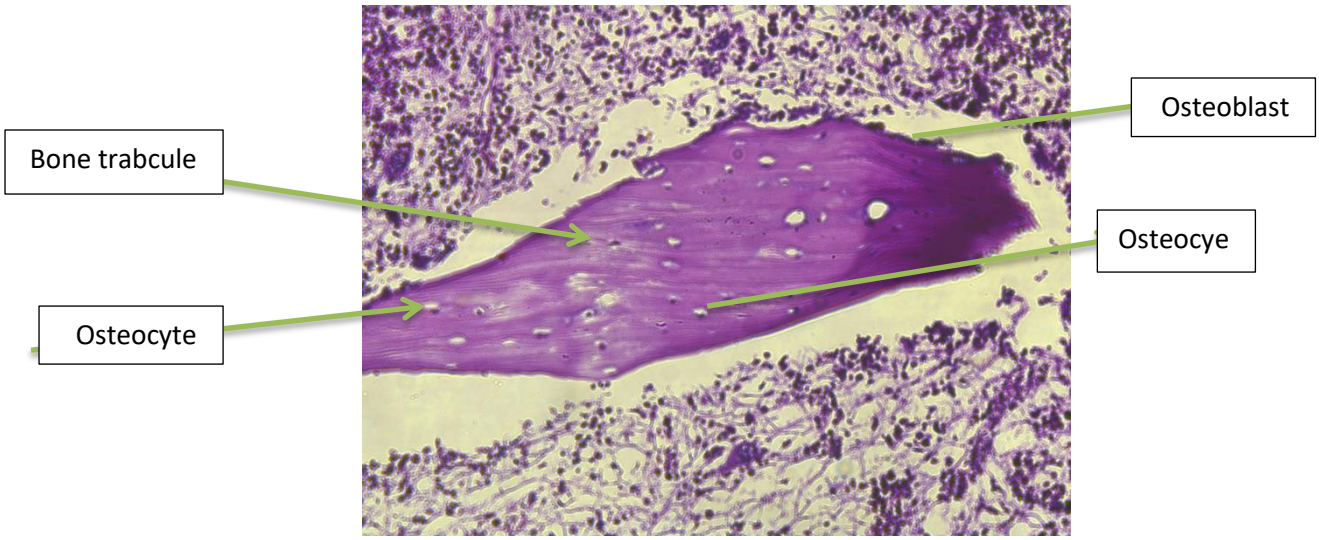



**Figure 3.22: Bone trabeculae inside the implant space (400x) after 2 weeks.**

**3.2.3.4 Histological feature of nano calcium sulfate coated screw without egg shell powder (group D).**

After 2 weeks of implantation, the microphotograph at the 40x magnification power showed osteoid tissue around the implant space **(Figure 3.23),** the bone trabeculae presented with small amount of osteocyte and with few number of scattered osteoblast within marrow elements **(Figure 3.24)**.

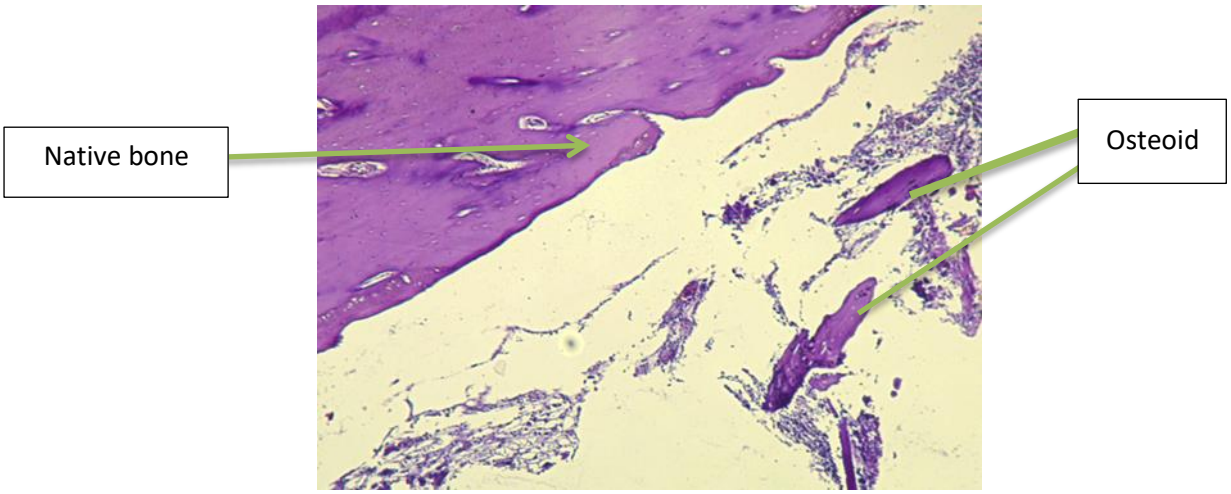



**Figure 3.23 Microphotograph of nano calcium sulfate dip coated with gap around implant not filled by eggshell powder (40x) after 2 weeks.**

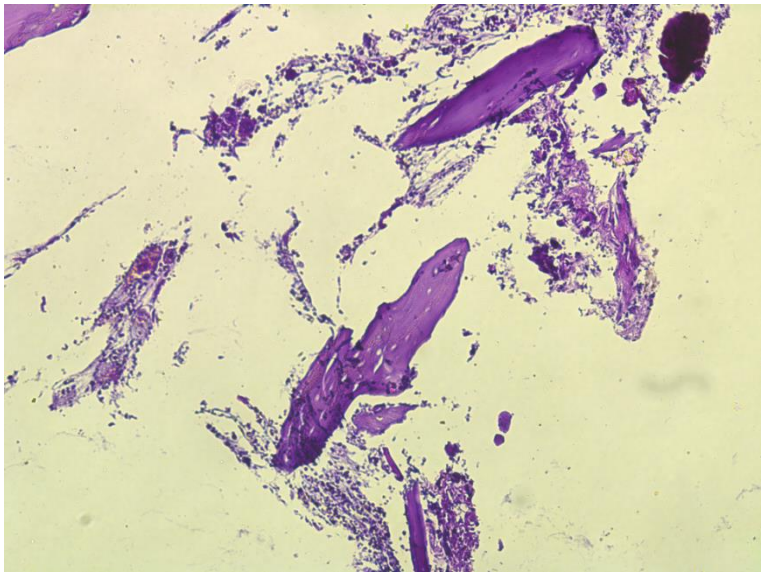


**Figure 3.24 Microphotograph of nano calcium sulfate dip coated with gap around implant not filled by eggshell powder (200x) after 2 weeks.**

## 3.2.4 Histological feature at 6 weeks of implantation

### 3.2.4.1 Histological features of the uncoated screw ( group A)

The microphotograph at the 40x magnification power showed bone trabeculae surrounding the implant space and at 400x magnification power these bone trabeculae surrounded by osteoblastic rim and fibrous tissue (**Figure 3.25 and Figure 3.26).**

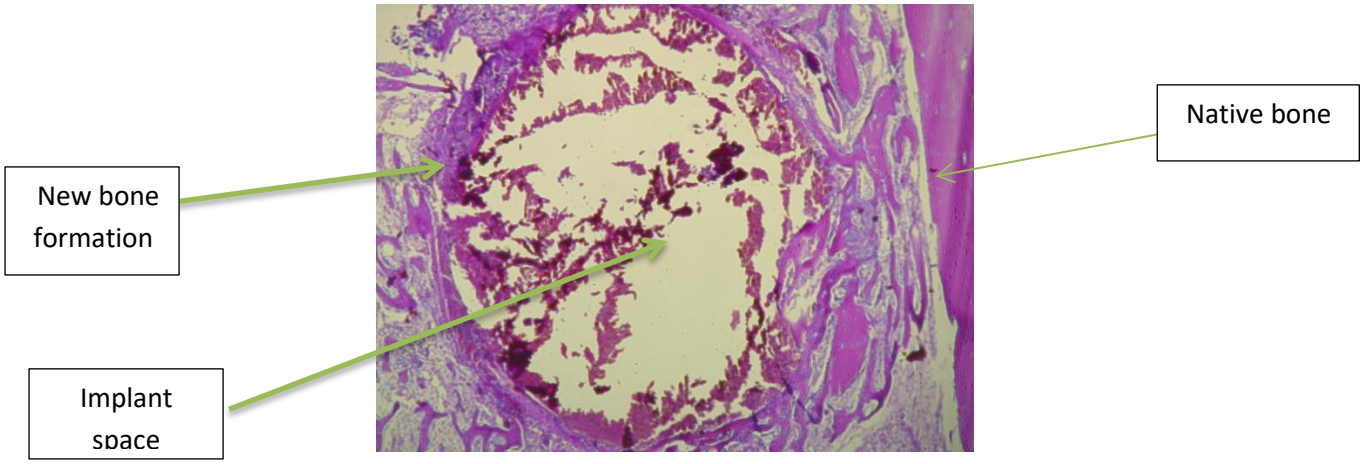



**Figure 3.25 Microphotograph of the control group after 6 week of implantation (40x) after 6 weeks.**

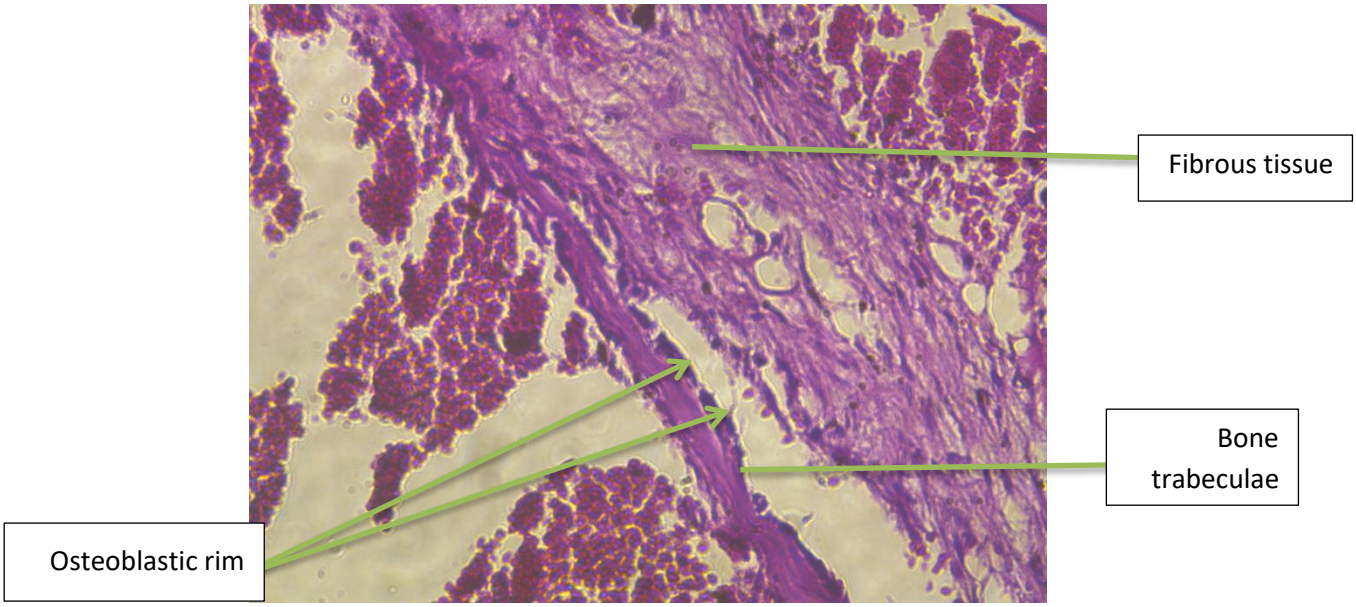



**Figure 3.26 Microphotograph of the control group after 6 week of implantation 400x after 6 weeks.**

### 3.2.4.2 Histological features of nano calcium sulfate coated screw only (group B)

The microphotograph at the 40x magnification power showed well defined bone trabeculae surrounding the implant space and at 400x magnification power these trabeculae in the thread regions presented with more calcified bone containing more numbers of osteocyte, reversal lines that separate between old and new bone and haversian system, surrounded by osteoblastic rim (**Figure 3.27 and Figure 3.28**).

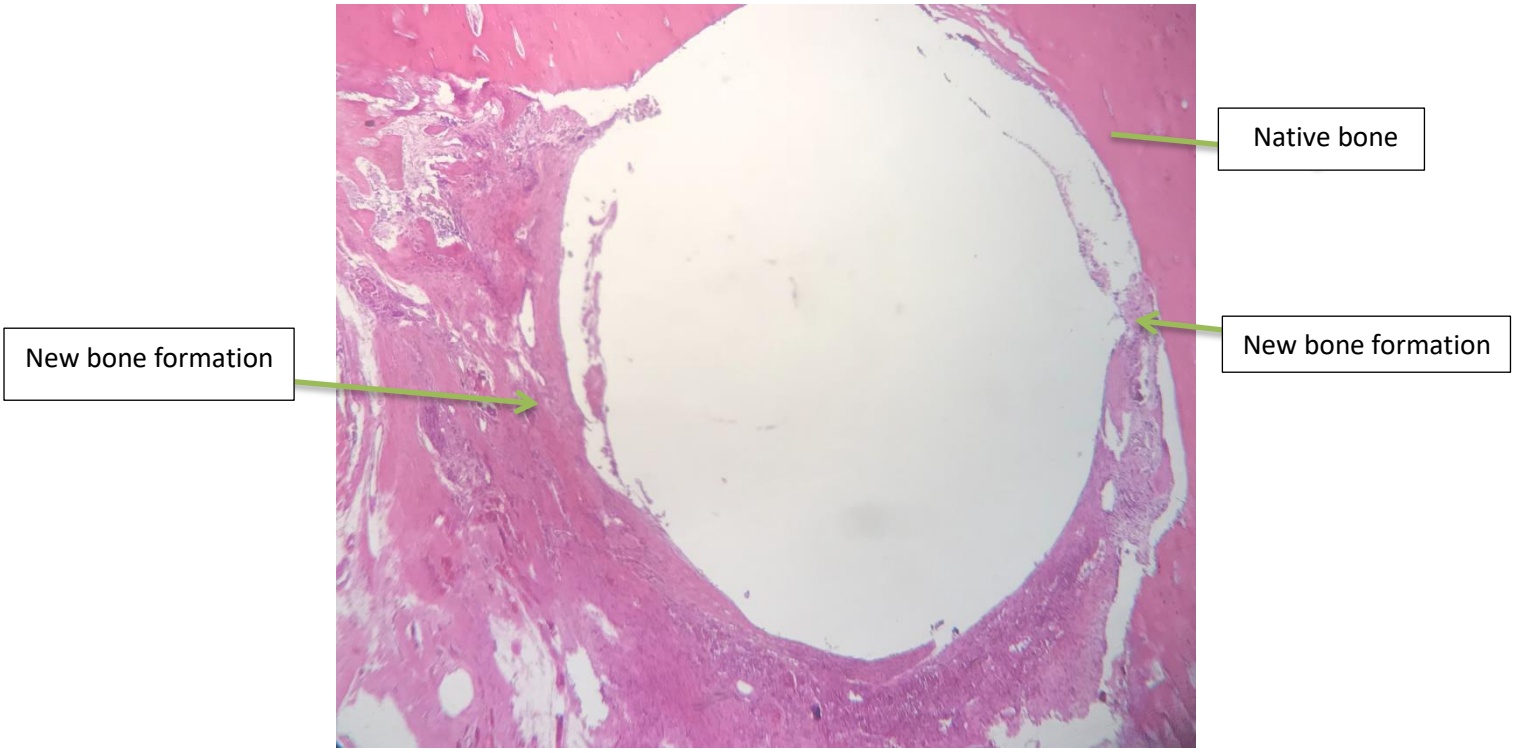



**Figure 3.27 Microphotograph of nano calcium sulfate dip coated (40x) after 6 weeks.**

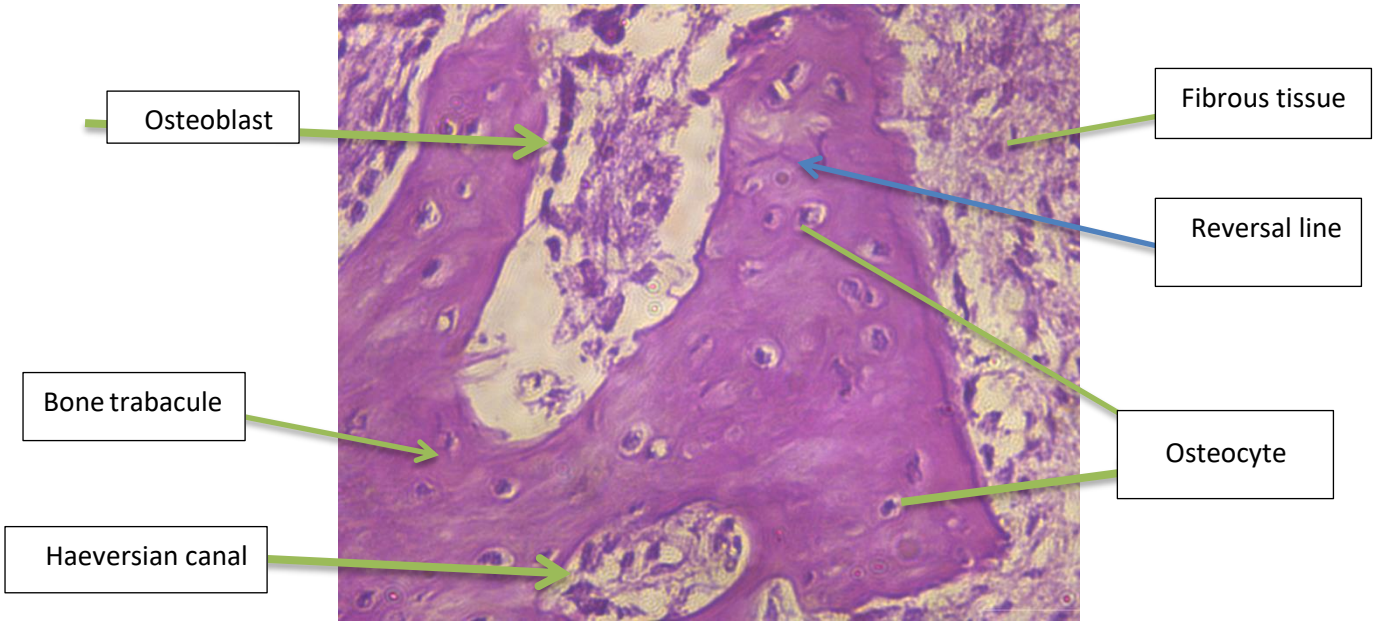



**Figure 3.28 Microphotograph in the thread region of nano calcium sulfate dip coated (400x) after 6weeks.**

### 3.2.4.3 Histological features of nano calcium sulfate coated screw with egg shell powder (group C).

The microphotograph at the 40x magnification power well defined and large amount of new bone trabeculae surrounding the implant space and at 200x magnification power these trabeculae in the thread region showed high number of osteocytes in lacunae that arranged in circular pattern around haversian system with scattered number of osteoclast,, The 400 x magnification power revealed well defined reversal lines and the osteoblast arranged in a rim surrounding the bone trabeculae **(Figure 3.29, Figure 3.30 and Figure 3.31)**

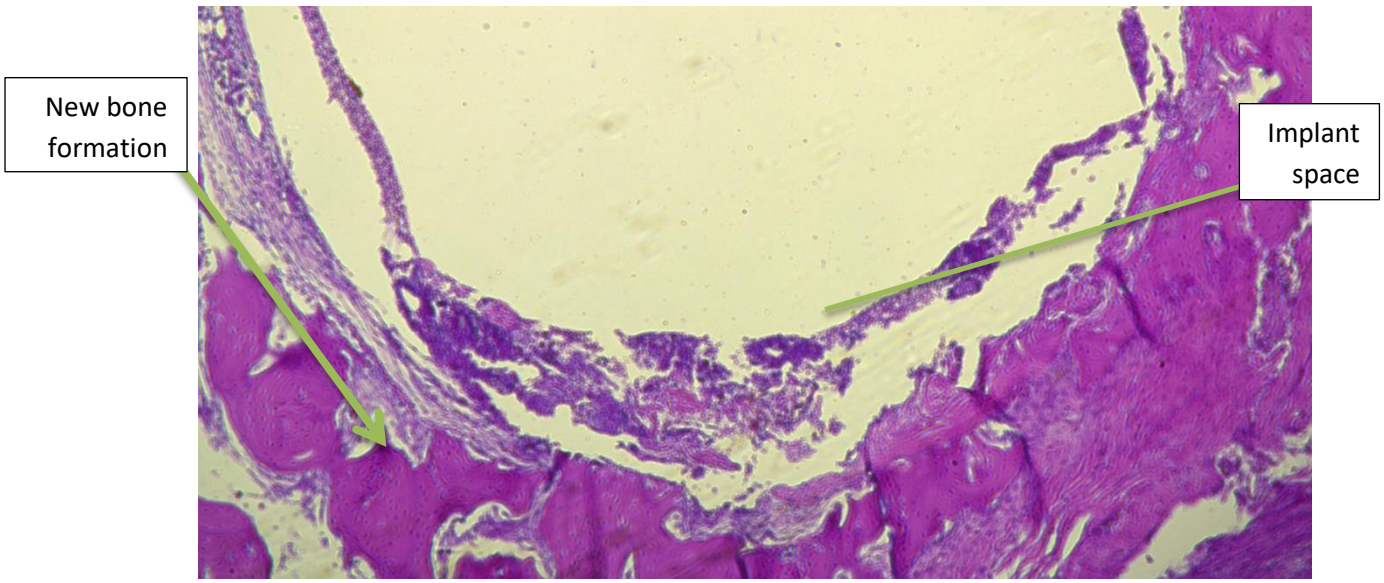



**Figure 3.29: Microphotograph of nano calcium sulfate dip coated with eggshell powder as graft material (40x) after 6 weeks.**

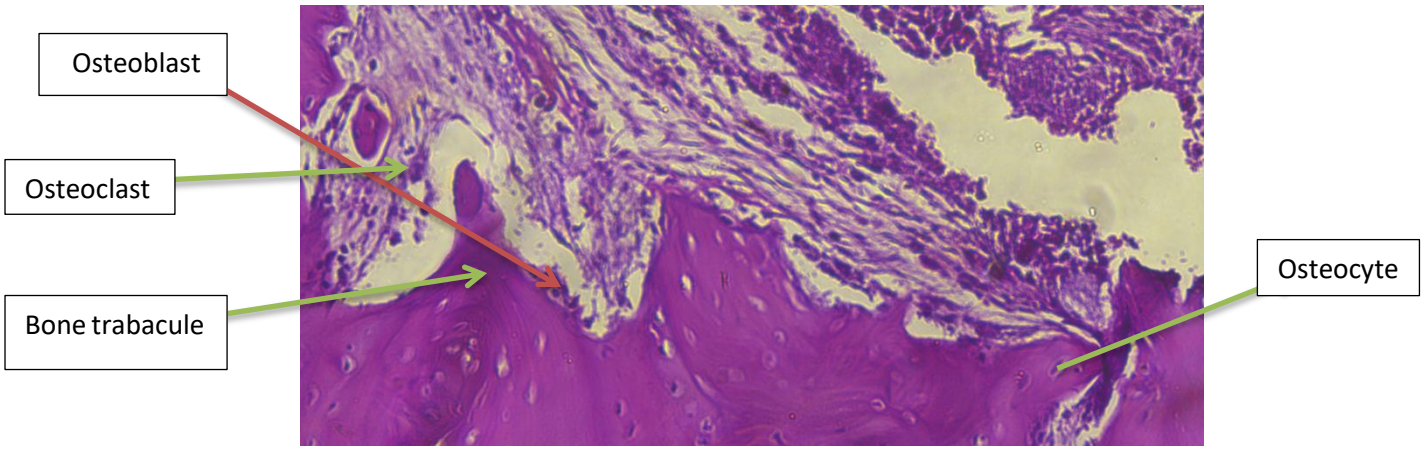



**Figure 3.30: Microphotograph in the thread region of nano calcium sulfate dip coated with eggshell powder as graft material (200x) after 6 weeks.**

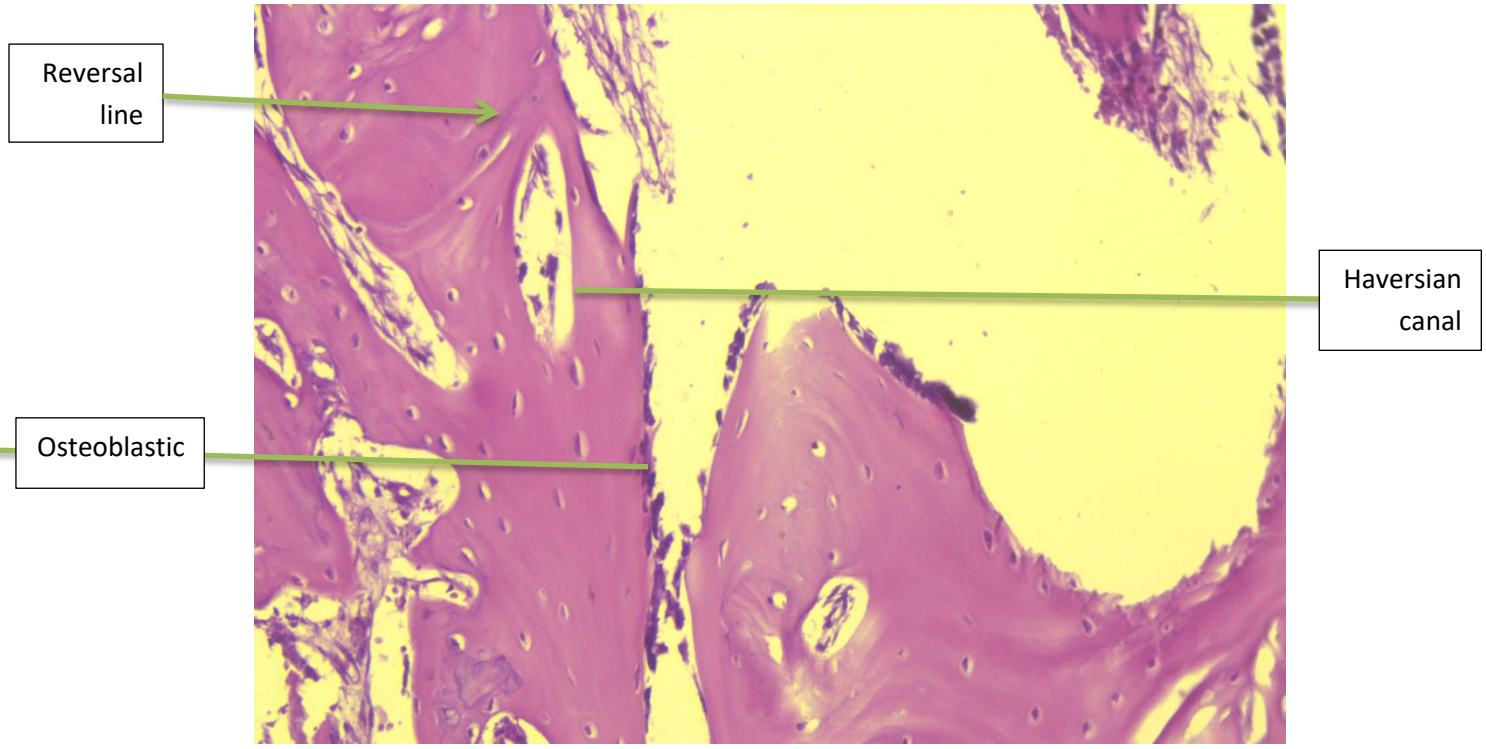



**Figure 3.31: Microphotograph in the thread region of nano calcium sulfate dip coated with eggshell powder as graft material (400x) after 6 weeks.**

### 3.2.4.4 Histological features of nano calcium sulfate coated screw without eggshell (group D)

After six weeks of implantation, the microphotograph at the 40x and 400 x magnification powers showed scattered bone trabeculae around the implant space with small number of osteocytes and surrounded by scattered osteoblast within marrow element **(Figure 3.31 and Figure 3.32)**

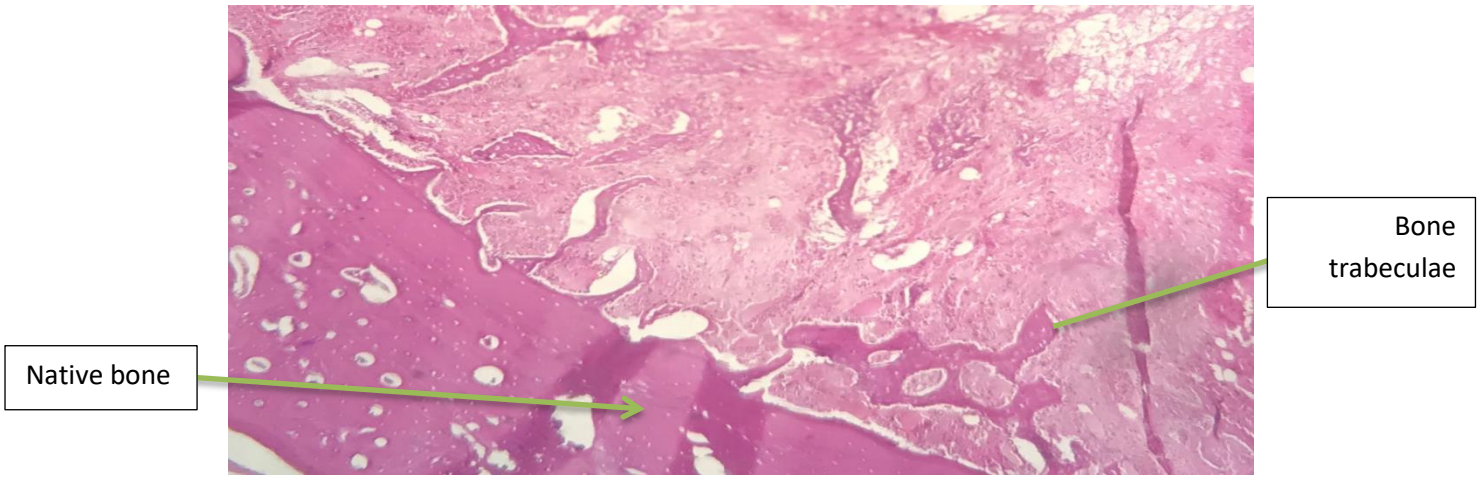



**Figure 3.31 Microphotograph of nano calcium sulfate dip coated with gap around implant not filled by eggshell powder (40x) after 6 weeks**

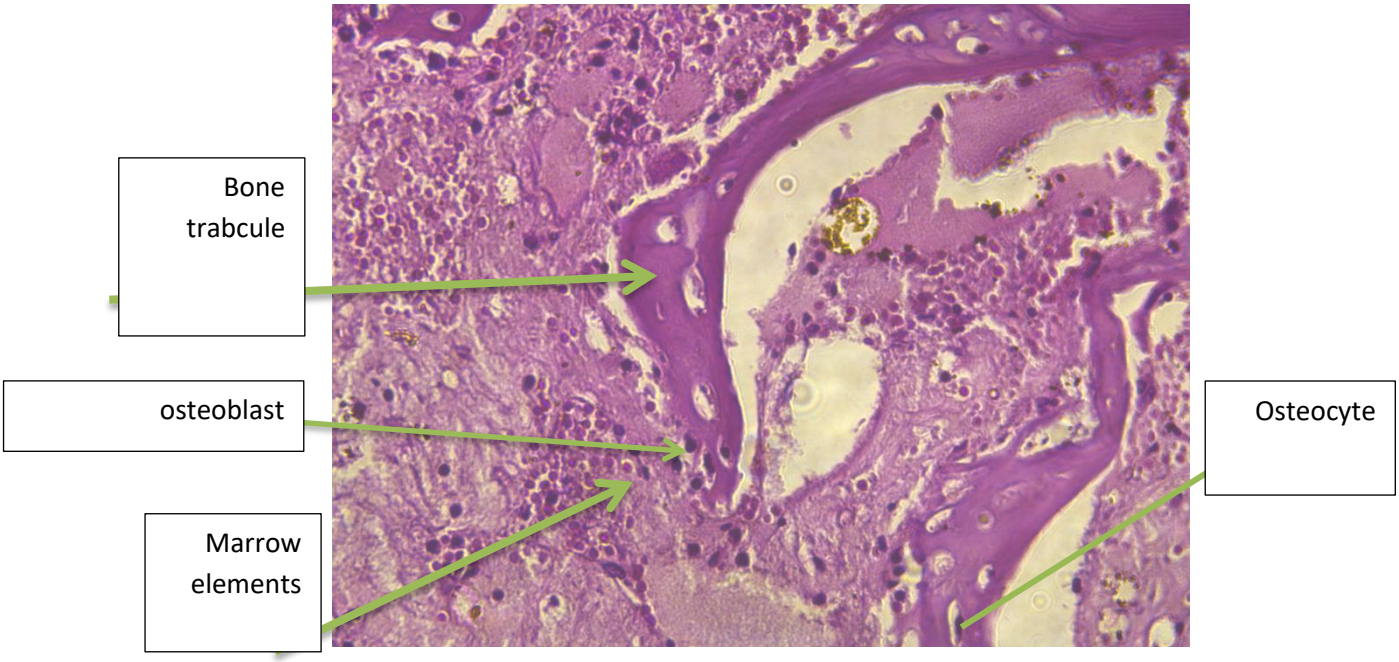



**Figure 3.32 Microphotograph of nano calcium sulfate dip coated with gap around implant not filled by eggshell powder (400x) after 6 weeks**

## 3.2.5 Histomorphometric analysis

In two weeks interval, the new bone formation percent (NBFP) of nano calcium sulfate dip coated with egg shell powder as graft material (**Group C).** It mean value was 0.46012% which was greater than remaining group as shown in (**Table 3.13).**

**Table 3.13 Descriptive statistic of percentage of bone formed among groups in the 2 weeks interval.**

| Period | | N | Mean | ±SD | ±SE | Minimum | Maximum |
|---|---|---|---|---|---|---|---|
| 2 week | A | 10 | 0.21430 | .080870 | 0.025573 | 0.110 | 0.390 |
| | B | 10 | 0.36194 | 0.098787 | 0.031239 | 0.234 | 0.507 |
| | C | 10 | 0.46012 | 0.117956 | 0.037301 | 0.301 | 0.610 |
| | D | 10 | 0.21012 | 0.030408 | 0.009616 | 0.149 | 0.246 |

To know if the sample have equal variances the levene's test is used for two week, since $P< 0.01$, levene's test is statistically highly significant and equal variance is not assumed as shown in **(Table3.14),** so in such case Games-Howell test for multiple comparison will be used.

**Table 3.14 Leven's test for new bone formation percent at 2 weeks.**

| Leven's Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| **5.500** | 3 | 36 | .003 |

The significance difference between groups for 2 weeks interval were tested by ANOVA as shown in **(Table 3.15).** It was found that there is statistically highly significant difference among the experimental groups $P< 0.01$.

**Table 3.15: ANOVA table of new bone formation percentage at 2 weeks interval.**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| **Between Groups** | **0.444** | **3** | **0.148** | **18.995** | **0.000 HS** |
| **Within Groups** | **0.289** | **36** | **0.008** | | |
| **Total** | **0.724** | **39** | | | |

Multiple comparison (Games-Howell) test at 2 weeks healing interval revealed that there is highly significant difference between the control group (A) and the third group(C),group (C) and group (D), control group (A) and group (B) , group (B) and group (D), $P< 0.01$ and non-significance between the remaining group at $P> 0.05$ shown in **(Table 3.16).**

**Table 3.16: Multiple comparison Games-Howell test of new bone formation percentage between groups at 2 weeks interval.**

| (I) Groups | | Mean Difference (I-J) | Sig. |
|---|---|---|---|
| **A 2w** | **B 2w** | **-.147640*** | .009 HS |
| | **C 2w** | **-.245820*** | .000 HS |
| | **D 2w** | **.004180** | .999 NS |
| **B 2w** | **C 2w** | **-.098180** | .219 NS |
| | **D 2w** | **.151820*** | .004 HS |
| **C 2w** | **D 2w** | **.250000*** | .000 HS |

In the six weeks of implantation interval, the new bone formation percentage (NBFP) of nano calcium sulfate dip coated with egg shell graft material group

(C) it mean value was 2.34464%, which was greater than other groups as shown in **table 3.17**.

**Table 3.17: Descriptive statistic of percentage of bone formed among groups in the 6 weeks interval.**

| Period | | N | Mean | ±SD | ±SE | Minimum | Maximum |
|---|---|---|---|---|---|---|---|
| 6 week | (A) | 10 | 1.31160 | 0.175342 | 0.055448 | 1.080 | 1.572 |
| | B | 10 | 1.91650 | 0.154180 | 0.048756 | 1.690 | 2.160 |
| | C | 10 | 2.34464 | 0.418851 | 0.132452 | 1.926 | 3.280 |
| | D | 10 | 1.20226 | 0.242432 | 0.076664 | 0.950 | 1.710 |

To know if the sample have equal variances the levene's test is used for two weeks, since the significant value is.025 which is less than 0.05, levene's test is significant so equal variance is not assumed as shown in **(Table3.18),** so Games-Howell test for multiple comparison was used.

**Table 3.18: Levene's test for new bone formation percent at 6 week interval.**

| Leven's Statistic | df1 | df2 | Sig. |
|---|---|---|---|
| **3.523** | 3 | 36 | .025 |

The significance difference between groups for 6 weeks interval were tested by ANOVA as shown in **(Table 3.19).** It was found that there is statistically highly significant difference among the experimental groups $P< .001$.

**Table 3.19: ANOVA table of new bone formation percentage at 6 weeks interval.**

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| **Between Groups** | **8.609** | **3** | **2.870** | **39.755** | **0.000 HS** |
| **Within Groups** | 2.599 | **36** | **.072** | | |
| **Total** | 11.207 | **39** | | | |

Multiple comparison (Games-Howell) test in 6 weeks healing interval revealed that there is highly significant difference between the group (A) and the group(C), group (A) and group (B), group (B) and group (D), group (C) and

group (D) $P< 0.01$ and significance between the group (B) and group (C), and non-significance between the remaining group $P> 0.05$ levels shown in (**Table 3.20**). A bar chart showed the summary of the difference in the new bone formation percentage value between all groups (**Figure 3.32**).

**Table 3.20: multiple comparison Games-Howell test of new bone formation percentage between groups at 6weeks interval.**

| (I) Groups | | Mean Difference (I-J) | Sig. |
|---|---|---|---|
| Control 6w | B 6w | -.604900* | .000 HS |
| | C 6w | -1.033040* | .000 HS |
| | D 6w | .109340 | .662 NS |
| B 6w | C 6w | -.428140* | .047 S |
| | D 6w | .714240* | .000 HS |
| C 6w | D 6w | 1.142380* | .000 HS |

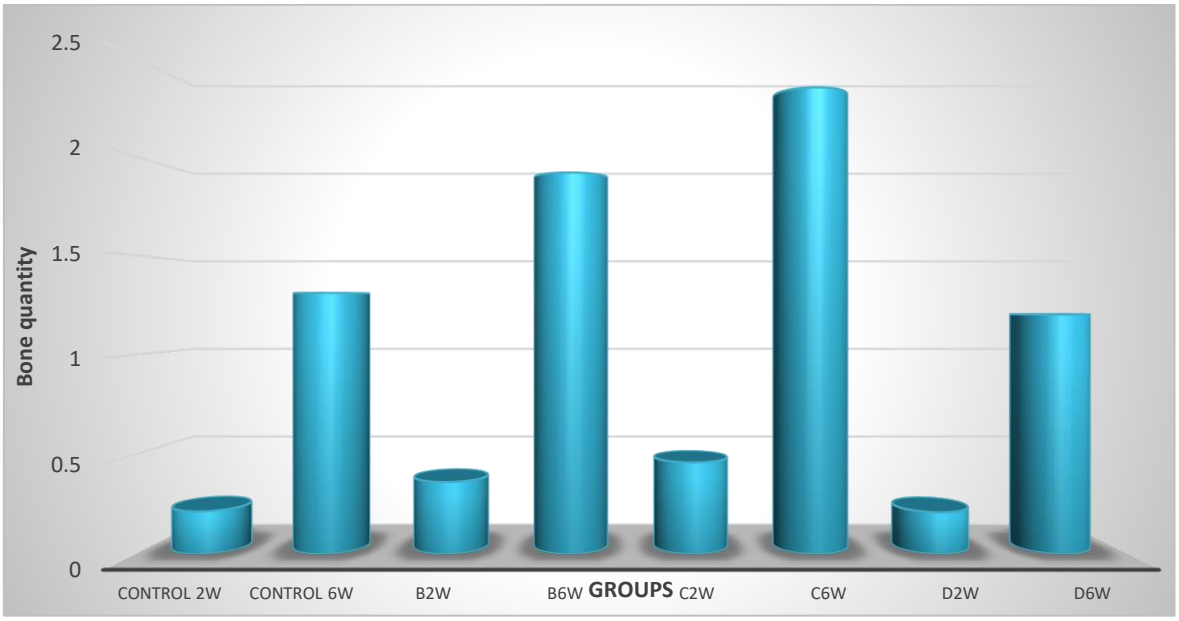



**Figure 3.33: A bar chart showed the summary of the difference in the new bone formation percentage value between all groups.**

# *Chapter Four*

# *Discussion*

# Discussion

This study concentrates on using nano calcium sulfate as a coating material for titanium implant screw by dip coating technique and applying egg shell powder on bone defect as bone graft material around coated titanium dental implant with nano calcium sulfate. And comparing the result mechanically, histologically and histomorphmetrically to the other groups which are (uncoated group, the coated group with no bone defect , coated group with bone defect filled by egg shell powder, coated group with bone defect not filled by egg shell powder).

Calcium sulfate work as bone grafting material for many years and have the ability to degrade rapidly between 4-6 week this feature minimize the use of calcium sulfate in defect with large size, so to solve this problem nano calcium sulfate has been emerged, which degrade almost in 12 weeks (**Tovar *et al.*, 2011).**

Calcium sulfate has many promising feature like (biocompatibility, easy to handle, angiogenic, osteoconductive, the fast entry of osteoprogenitor cell to the defect site as compared to other bone graft material and no inflammatory response **(; MacNeill; *et al.*, 1999 ; Fujishiro *et al.*, 2007; Ricci *et al.*, 2008).** So in this study nano calcium sulfate was chosen to evaluate it's effectiveness as coating material for Cp Ti as no published paper can be found studying nano calcium sulfate as a coating material.

In this study PVA which work as coating and binding agent secure cohesion to the coating layer **(Moreau *et al.*, 2014)** and used to enhance the fluidity state of powder **(Su *et al.*, 2018),** PVP also work as coating and binding agent, moreover it enhance the flexibility and plasticity and decrease brittleness (plasticizers) and have high density and strength **(Kim *et al.*, 2007).**

Calcium sulfate set more quickly with blood, so calcium sulfate that is preset should be applied as bone graft material it dissolve more consistently and slowly than calcium sulfate that use blood for setting. In bone region usually it's preferable to work in dry condition to reach good setting. A good setting in calcium sulfate is achieved using NaCl which accelerate the setting of calcium sulfate **(Pecora *et al.*, 1998 ; *De* Leonardis, *et al.*, 2000)**

## 4.1.1 Coating characterization:

### 4.1.1.1 Optical microscopical observation and surface morphology (SEM)

The optical microscopical observation result for the dip coated nano calcium sulfate using PVA (Figure 3.1) showed that nano calcium sulfate uniformly distributed and dense on the titanium disc with holes on coating film, while the dip coated nano calcium sulfate using PVA and PVP showed that nano calcium sulfate coated layer uniformly distributed and dense on the titanium disc with less number of holes on coating film.

Morphological analysis, by using SEM for Cp Ti disc coated with nano calcium sulfate with (PVA+PVP) as a binding agent has confirmed the optical microscopical result for dip coated disc, beside, it clarify the blending of small poly crystalline structure over each other forming a bigger poly crystalline structure size **(Figure 3.11).**

No cracks appears on optical microscope and SEM analysis in the coated disc may be due to less shrinkage environments in the coated layer of nano calcium sulfate **(Nasir and Rhaman, 2016; Zwain and Hamad, 2018)**. Holes appear as a result of liquid phase evaporation which related to the solution type and the coating film thickness. Or due to incomplete spread of particle. This explanation agreed with (**Su *et al.*, 2018 ; Santillan *et al.*, 2010)**.

### 4.1.1.2 Atomic force microscope observations

In this study, the coated discs showed coating film that have nano-rough surface, **(Figure 3.11).** The nano calcium sulfate solution appear to have the most effective result when used with (PVA+ PVP) as binding agent when compared to nano calcium sulfate solution that use only PVA as binding agent, The two coated disc has approximately the same granulation distribution with more uniform distribution of grains in (PVA+PVP) and surface roughness for (PVA+PVP) as binding agent was (3.28 nm) while for PVA alone was (2.53 nm), so (PVA+PVP) was chosen so that the surface area increased and increased in surface roughness increased on implant surface that promote bone apposition this agree with the finding of **Albrektsson and Wennerberg in 2004**.

### 4.1.1.3 Phase identification

It is obvious from XRD schemes **(Figure 3.6 and Figure 3.7),** that the surface of the disc was well coated with nano calcium sulfate using PVA alone and (PVA, PVP) as binding agent respectively, because most of the diffraction peaks could be indexed to calcium sulfate matching with (ICDD 37-1496) for calcium sulfate in peaks ( (111), (020), (121), (040) and (224) ). The presence of titanium peaks in the XRD pattern after each coating process was due to the penetration of X- rays beyond the coated layer.

The narrower peaks of XRD are related to highly crystalline nature of layers, while broad peaks showed decreased levels of crystallinity, this explanation in agreement with (**Gruene, 2014**).

It is obvious from the XRD schemes of egg shell for with particle size (50 µm,150µm**)** particle size **(Figure 3.8 and Figure 3.9)** showed that eggshell it m mainly composed of calcium carbonate because most of the diffraction peaks could be indexed to calcium carbonate matching to with (ICDD 29-0305 ) in peaks (002), (112), (202), (112), (013), (032) for (50 µm) sample. This is naturally happened because the egg shell powder need more heat treatment than

that heat used for sterilization of egg shell powder to reach identical match and perfect crystallization . And the same peaks for for150µm except (821) and there was a little shift in peaks (302) and (204) this is due to bigger size of particle, because the particle size was bigger than before, so the XRD can detect the calcium carbonate more than before.

These findings was agreed with that found by **(Krithig *et al*., 2011; Anjaneyulu *et al.*, 2014)** which they found the calcium carbonate is the major component in the XRD of egg shell particle.

**4.1.1.4 Microhardness test**

Vickers microhardness test for the uncoated and coated nano calcium sulfate discs indicates that the average Vickers hardness (HV) numbers are (230.4 for the uncoated group), (237.6 two coated layer), (248.6 three coated layer), (254.3 four coated layer) as shown in **(Table 3.1)**.

These changes in Vickers numbers are appeared because of the variation of surface topography between the coated and uncoated titanium discs, while in coated nano calcium sulfate groups the increased in number of coating layer will lead to increase in film thickness, consequently Vickers hardness number increased. These explanation was agreement with (**Santillan *et al.*, 2010; Greer *et al.* 2016,)** where they found that the diversity in sintering environment and coating composition are affect the microhardness value of titanium coating surface.

**4.1.1.5 Elemental composition**

EDX analysis in (**Figure 3.11 and Figure 3.12)** of the uncoated disc and the coated nano calcium sulfate groups showed that the mutual component in the surface of the all discs was O, the coated nano calcium sulfate showed increased surface oxide percentage as compared to uncoated one due to oxygen content in calcium sulfate hemihydrate this finding in confirm with **(Kuthadi, 2014)** or because of increasing in titanium dioxide film as stated by **Hammouda *et al.* (2014)**, who found in (EDX) plot the increase in oxygen

percentage is connected to increasing in titanium dioxide that formed on Cp Ti disc. Also the increase of oxygen in percentage titanium may be related to oxidation as consequence of high temperature of sintering this agrees with **Anish *et al.* (2013)**. The appearance of carbon in (EDX) analysis of coated titanium disc, because carbon considered as dielectric fluid with ruminants usually contains carbon as revealed by **Anish *et al*. (2013)**. In (EDX) mapping confirm the uniform distribution of particle. And the main element of coated disc are (O, S, Ca) this result agree with the finding revealed by **(Kuthadi, 2014).**

#### 4.1.1.6 FTIR analysis for eggshell powder

The FTIR result of egg shell particles confirm that calcium carbonate is the main component, The peaks of 50 μm at 710, 876,1433, 1799, 2515, 2873 indicate the $CaCO_3$ is the major components, the peaks of absorptions that appears at: 2873,2979,1799 are related to $CO_3^{-2}$, the most strong peak is 1433 which correspond to C-O while the peak 3288 is related to O-H group this explaination agree with the findings of **Aldrich, 1997; Krithiga, 2009; Guru *et al*., 2012; Antanyulu, 2014.** And for egg shell powder with particle size of 150μm has the same peaks variation.

## 4.2 In vivo experiments

### 4.2.1 Experimental animal description

Adult male New Zealand white rabbits were chosen because of various reason which are rapid osseointegration rate, no difficulties in controlling these rabbits, availability and economically cheap in relation to other animals, as well as when compared to other species, these strains are considered to have minimum aggressive nature with low ratio of health problem. According to these reasons many implant reports have been used the rabbit tibia as successful

implantation site **(Shkur, 2014; Jani, 2014; Hussein, 2015, Refaat, 2016 and Azzawi *et al.*, 2018)**.

In this study, the age of these rabbit approximately ranged from (10-12) months because at this age the proximal rabbits tibia is resemble to adult human bone physiologically as stated by many authors **(Michaels, *et al.* , 1997; Refaat *et al.*, 2016)**.

The tibia area of the rabbits was chosen to reduce the clinical situation, as the dimensions of this bone almost resemble the human alveolar space. Tibia is considered as a suitable implant location site for various reasons: which in surgical part ; low level of morbidity with an approaching the medial proximal tibia for placement of the implant and anatomically with an ease ; as presence of cancellous bone by working as cushion and avoid splinting of cortical bone, these facts stated by **Dahlin *et al.* (1989)**, **Mapara *et al.* (2012)** and **Alkhafaji (2015)**

**Dupoirieux *et al., in 2001*** found that egg shell with the particle size of 50μm lead to fast healing process of bone as compared to the other larger particle , so in this study egg shell with particle size of less than 50μm was chosen as bone graft material.

## 4.2.2 Radiographical examination

The radiographic evaluation applied for presenting contact among bone and implant, At the end of both the two and six weeks healing period , the radiographical evaluation **(Figure 2.36 and Figure 2.37)** clarify that no radiolucent regions were detected or any abnormality activity from bone to the implant **Atsumi, *et al.*, 2007.** Explain that it is difficult for a implantologist to diagnose the radiographic bone loss at 0.1mm resolution, so no radiolucency in the implantation site is not a sign for osseointegration.

## 4.2.3 Clinical tests

The primary and secondary stability for all implants are evaluated on the basis of mobility detection through instrument with blunt end, this test reveal whether the implant is stable or not, after implant insertion and also at the end of the healing intervals. This is done by implant resistance to removal by manual force without the help of torque meter device. These evaluations are considered the requirement of nano calcium sulfate coated Cp Ti to be successful. These results similar with that of **Mistry *et al. in* 2014** and **Smeets *et al.* in 2016**, who demonstrated that the achievement and preservence in implant stability are regarded as requirements for successful implants.

Gamma irradiation was used in this study for both uncoated and coated implants. The purpose of applying irradiation sterilization rather than autoclaving to remove contamination of surface from accumulation of the salts that may be available in autoclaves steam **(Benson, 2002). Silindir in 2009** suggest that sterilization of the implants by gamma irradiation produce an obvious surface when compared with various sterilization method (autoclaving and exposure to UV- light), as it was confirmed by the increase in the thickness of the oxide layer and reduce of the wetting angles. Gamma sterilization has no negative affect the surface composition of the titanium disc studied by **Sitting *et al.,in* 1999**.

No sign of any severe infection in the implantation area, after each interval, may reveal that the material is tolerated, an excellent conditions for implantation involve implant sterilization and instrument autoclaving. The surgery was done in a septic to environment to avoid the gross infection, the use of sharp drills during the surgery precisely with cooling and discontinuous pressure to prevent overheating of bone and distribution of necrotic bone in the bed of implant then establishment for osseointegration.

## 4.2.4 Mechanical test

Implant resistance to torque removal has been related with the level of bone-implant contact by many reports, that’s connect the alteration in

biomechanical feature of regeneration process and dynamics of bone- interface healing **(Faeda *et al.*, 2009).**

Torque is the movement or twisting applied by load at a span on body identical with load multiplied by the horizontal span between the center of rotation and line of action at which its applied **(Yousef*et al.*, 2005).**

The removal torque used in this study is considered as sign for the availability of osseointegration and work as mechanical properties tester of bone implant interface.

The removal torque described as the torsional force important for removal the implant fixture, the removal torque value was measured using a digital torque meter in Newton centimeters (N/cm). This technique primarily imposed on interfacial shear properties. Moreover, the result may be related to implant geometry and topography as stated by **(Yeo *et al*., 2008 ; Chang, *et al*., 2010).**

In this study, removal torque values was found to be significantly increased in the dip coated only (group B) and dip coated with bone graft around implant (group C) with time **(Table 3.8)**, this might be because consequent bone formation in implant- bone coating contact as well as due to bone remodeling around the implant during the healing period that consequently improve the mechanical capacity with time. Bone graft insertion with implant simultaneously reduce healing interval with no risk of raising complication or minimizing the success rate as found by (**Boronat *et al.*, 2010).**

This is also can be related to bioactive mechanism of calcium sulfate bone formation as calcium sulfate implanted inside the bone defect and contacted the body fluid leading to dissolution to calcium and sulfate ions, phosphorus ions that released from body fluid combine with calcium ion forming calcium phosphate at the surface of calcium sulfate that's considered as self-forming biologic appetite, calcium sulfate is soluble in body fluid slowly leading to deposition of carbonate apatite that has stability under same environments and going to be positioned back then transformed to bioactive

elements which induce bone formation, these explaniation was agreed with that found by (**Ricci *et al.*, 2000**). This explain the highly significance difference of (group B) paired t-test comparison as shown in **(Table 3.12).**

Also calcium sulfate combine with other graft material leading to raise bone production, better handling characteristic, improve molecule graft containment, this will lead to more bone production to the defected site as compared to allograft material alone, so when used with egg shell bone graft material which more quantity of bone regeneration as compared to unfilled defect by increasing the quantity of mineralized new bone due to showing biodegradable feature which change graft particle by new bone these result agreed with (**Al ruhaimi, 2000; Uraz, *et al.*, 2013)** which they use different alloplastic bone graft material **.** This explain the significance different of (group C) paired t-test comparison as shown in **(Table 3.12),** which reveal that bond intensity of implant-interface has increased, beside this as mentioned by (**Mistry *et al.*, 2014)** the mechanical test can not be used to verify the osseointegration. That's the reason for using histological analysis in this study to ensure the osseointegration of each group.

## 4.2.5 Histology and Histomorphometry analysis

Implant stability can be evaluated using histological analysis which can be done at any time of implantation as mentioned by (**Atsumi *et al.*, 2007).**

In this study the result showed that osseointegration can be achieved as implant placed in a vital bone with convenient biological condition for bone generation is made, the bond strength increased between osseointegrated implants and bone by time as healing and bone remodeling continue, also the level of bone implant has increased and development of bone appears, this characteristic was in coincidence with **Liu *et al.*, in 2004.**

In this study the result showed that nano calcium sulfate has satisfactory osteoconductive capability and acceptable biocompatibility, as calcium sulfate degrade it deposit calcium phosphate at bone implant contact so adherence of

osteoblast to calcium phosphate rapidly and more bone deposition, these finding was also seen when calcium sulfate used as bone graft material by (**Riccci, 2008).**

After two weeks of implantation the implantation site for all groups in the rabbit tibia (**Figure 3.17 Figure 3.19, Figure 3.21 and Figure 3.23)** showed the presence of bone trabeculae that have numerous osteocyte and rimmed by osteoblast, beside this the coated nano calcium sulfate (group B), and coated nano calcium sulfate with egg shell as bone graft material (group C) showed more bone trabeculae in the implant space that contain increased number of osteocyte and the bone trabeculae rimmed by osteoblast as compared to the remaining groups.

After six weeks of implantation the implantation site for all groups **(Figure 3.25, Figure 3.27, Figure 3.29 and Figure 3.31)** showed bone trabeculae and beside that the coated nano calcium sulfate (group B) showed thread region with calcified bone containing more numbers of osteocyte, reversal lines and haversian system, surrounded by osteoblastic rim. Group B show highly significance difference for removal torque value and histomorphometric analysis (NBFA) in 2 and 6 weeks of implantation and being the second biggest mean value for removal torque and (NBFA) in 2 and 6 weeks of implantation.

The coated nano calcium sulfate with egg shell as bone graft material (Group C) showed in the thread region high number of osteocytes in lacunae that arranged in circular pattern around haversian system with scattered number of osteoclast, The 400 x magnification power revealed well defined reversal lines and the osteoblast arranged in a rim surrounding the mature bone trabeculae as shown **(Figure 3.30 and Figure 3.31)** which is the best result obtained in this group where maturation and mineralization of bone at thread regions was observable in 2 and 6 weeks intervals. These findings for group C confirmed by the results of mechanical test (torque removal test) and histomorphmetric analysis which recorded higher mean values for new bone

formation area (NFBA) and torque mean value for both 2 and 6 weeks intervals as compared to other groups and this may be explained as the combined effect of nano calcium sulfate for bone generation which is conversion of calcium sulfate as it dissolve in body fluid to calcium phosphate that increase adherence of osteoblast to the defect site as stated by **(Ricci *et al.*, 2008**) and calcium sulfate may cause local reduction of PH which is related to bone mineralization that lead to release of osteoinductive molecule inside bone matrix activating healing stage as found by **(Borreli and Evaniew, 2013).** The ability of calcium sulfate to work with other bone graft material (egg shell) that used in this study as binder which improve production in the defected site, this explaination was conform with the study of **(Al ruhaimi, 2000)** who showed the ability of calcium sulfate to bind with other material, so bone production increased due to calcium sulfate effect and calcium carbonate derived from egg shell has been described to be effective as a osteoconductive and biocompatible biomaterial in many experimental animal studies **(Dupoirieux *et al.*, 2001; Durmus, *et al.*, 2003).**

The histological results in this study showed formation of new bone around coated implants without fibrous encapsulation or inflammatory reaction during the experimental interval regardless the implant coating material and the duration of the implantation. In spite the implants material difference , techniques, and shapes of implant used, still the result of this study agree with the findings of **(Jani, 2014; Hussein, 2015; Hamad *et al.*, 2018).**

The distinction in the quantity of bone tissue produced around coated implant in the marrow space and in the cortical regions were significant proposing that nano calcium sulfate has high osteoconductivity after it application with egg shell as bone graft material.

# *Chapter Five*

# *Conclusions and Suggestions*

# Conclusion and Suggestions

## 5.1 Conclusion

The conclusion of this study are:

1- A dip coating technique can successfully make nano calcium sulfate as coating material for Cp Ti with the presence of (PVA+PVP) as a binding agents, as homogenous polycrystalline structure of calcium sulfate nano particle on the titanium surface and increase in surface roughness.

2- Egg shell of less than 50μm particle size was used as a bone graft material beside the nano calcium sulfate coated titanium implant successfully .

3- Histologically, the use of egg shell around nano calcium sulfate coated Cp Ti promote osseountegration better than other groups.

4- Histomorphometric analysis (NBFA) showed highly significance difference in 2 and 6 weeks of implantation and being the most highest mean value for both removal torque and (NBFA) in 2 and 6 weeks of implantation. Was found in nano calcium sulfate coated screw with egg shell powder around it.

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## 5.2 Suggestions:

Further studies suggestion:

1-Biomechanical evaluation for long term survival (8, 12 weeks) of nano calcium sulfate coated implants by the same coating method applied in this study.

2- Evaluation of nano calcium sulfate coated implants and using different bone graft material around it.

3- Applying different coating technique like plasma spray or laser as a coating method for calcium sulfate nano particle on Cp Ti.

4-Studying the biocompatibility test like cell culture.

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# *Appendices*

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

| No. | Control group | Coated nano calcium sulfate only | Coated nano calcium sulfate and gap around them filled by egg shell powder | Coated nano calcium sulfate and gap around them not filled by egg shell powder |
|---|---|---|---|---|
| **1** | 9 | 13 | 15 | 10 |
| **2** | 7 | 9 | 14 | 9 |
| **3** | 8 | 9 | 16 | 11 |
| **4** | 10 | 12 | 13 | 12 |
| **5** | 11 | 11 | 12 | 10 |
| **6** | 7 | 8 | 10 | 8 |
| **7** | 9 | 11 | 12 | 9 |
| **8** | 8 | 11 | 12 | 9 |

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

| No. | Control group | Coated nano calcium sulfate only | Coated nano calcium sulfate and gap around them filled by egg shell powder | Coated nano calcium sulfate and gap around them not filled by egg shell powder |
|---|---|---|---|---|
| **1** | 11 | 17 | 14 | 11 |
| **2** | 10 | 10 | 12 | 10 |
| **3** | 15 | 11 | 17 | 12 |
| **4** | 11 | 14 | 18 | 14 |
| **5** | 12 | 12 | 23 | 16 |
| **6** | 13 | 10 | 16 | 9 |
| **7** | 14 | 12 | 20 | 10 |
| **8** | 11 | 12 | 19 | 10 |

## NEW bone formation area 2 weeks

| No. | Control group | Coated nano calcium sulfate only | Coated nano calcium sulfate and gap around them filled by egg shell powder | Coated nano calcium sulfate and gap around them not filled by egg shell powder |
|---|---|---|---|---|
| **1** | 0.11 | 0.459 | 0.332 | 0.245 |
| **2** | 0.14 | 0.236 | 0.318 | 0.236 |
| **3** | 0.16 | 0.332 | 0.6011 | 0.2122 |
| **4** | 0.17 | 0.316 | 0.5211 | 0.185 |
| **5** | 0.19 | 0.3422 | 0.61 | 0.149 |
| **6** | 0.211 | 0.351 | 0.415 | 0.219 |
| **7** | 0.24 | 0.234 | 0.563 | 0.246 |
| **8** | 0.26 | 0.3356 | 0.517 | 0.211 |
| **9** | 0.272 | 0.5066 | 0.301 | 0.184 |
| **10** | 0.39 | 0.507 | 0.423 | 0.214 |

## NEW bone formation area 6 weeks

| No. | Control group | Coated nano calcium sulfate only | Coated nano calcium sulfate and gap around them filled by egg shell powder | Coated nano calcium sulfate and gap around them not filled by egg shell powder |
|---|---|---|---|---|
| **1** | 1.225 | 2.16 | 1.9478 | 1.05 |
| **2** | 1.11 | 1.923 | 2.271 | 1.0076 |
| **3** | 1.26 | 1.69 | 2.024 | 0.95 |
| **4** | 1.34 | 1.715 | 3.28 | 1.299 |
| **5** | 1.563 | 1.844 | 1.926 | 1.14 |
| **6** | 1.08 | 1.79 | 2.19 | 1.71 |
| **7** | 1.315 | 1.951 | 2.67 | 1.53 |
| **8** | 1.184 | 2.055 | 2.54 | 1.099 |
| **9** | 1.572 | 2.03 | 2.512 | 1.095 |
| **10** | 1.467 | 2.007 | 2.0856 | 1.142 |

# الخلاصه

**الخلفيه**

استعمال زرعات الاسنان في المناطق الضعيفه للعظم كما في المنطقه الخلفيه للفك العلوي مما يؤدي إلى زيادة فشل الزرع في هذه المناطق , تستخدم كبريتات الكالسيوم كماده للتطعيم العظمي لقدرته على التجديد العظمي لذلك في هذه الدراسه تم استخدامه كمادة طلاء لزرعات التيتانيوم التجاريه النقيه بالاضافه لقدرة كبريتات الكالسيوم للارتباط مع مسحوق قشور البيض الذي يعتبر ماده للطعوم العظمية حول هذه الزرعات لتحسين نوعية وكمية العظم في المناطق ذات المستوى المنحفض النوعيه للعظام.

**أهداف الدراسة**

تقيم تقنية الغمر لزرعات التيتانيوم التجاريه النقيه المطليه بكبريتات الكالسيوم النانويه, وتقيم ومقارنه ثاثير اضافة مسجوق قشور البيض كبديل عظمي على مناطق ذات عيوب عظميه حول زرع التيتانيوم النقيه تجاريا المطليه بكبريتات الكالسيوم النانويه ميكانيكيا عن طريق اختبار إزالة عزم الدوران ، نسيجيا و عن طريق قياس نسبة تكوين العظم داخل النسيج.

**المواد والادوات:**

تم تقدير خصائص طلاء طبقة من كبريتات الكالسيوم النانويه التي تشكلها تقنية طلاء الغمر على أقراص التيتانيوم النقيه تجارياً ذات الدرجه (II) باستخدام عدة مواد رابطه (كحول عديد الفينايل و بولي فاينيل بيرليدون) تم تقديرها من خلال: المجهر الضوئي ، تحليل حيود الأشعة السينية، مجهر القوى الذرية ، المجهر الإلكتروني الماسح ، تحقيقات الأشعة السينية المشتتة للطاقة وقياسات فيكرز الصغرى. ثم تقييم مسحوق قشور البيض من خلال تحليل حيود الأشعة السينية و ومطيافية الاشعه تحت الحمراء.

فيما يتعلق بالتجارب في الجسم الحي ، تم استخدام ٨٠ زرعه مصممة لزراعة الأسنان في عظام 20 من أرانب نيوزيلندا البيضاء. ٢٠ زرعة تبقى غير مطليه كمجموعة تحكم و الستين زرعه المتبقية المطليه بجزيئات كبريتات الكالسيوم النانويه، ٢٠ زرعه مطليه بواسطة كبريتات الكالسيوم النانويه تستخدم لوحدها في عظام الساق، ٢٠ زرعه أخرى مطلية بواسطة كبريتات الكالسيوم النانويه والفجوة التي صنعت حولهم مليئة بمسحوق قشور البيض كمادة الطعوم العظمية ، والزرعات ال ٢٠ المطليه الأخيرة بواسطة كبريتات الكالسيوم النانويه والفجوة التي صنعت من حولهم ولا تملؤها مسحوق قشورالبيض.

وأخيرًا ، تم تقدير الزرعات ميكانيكيا (من خلال اختبار عزم الدوران) وبيولوجيًا (من خلال الفحص النسيجي و قياس نسبة تكوين العظم داخل النسيج) بعد فترات الشفاء لمدة 2 أسبوعين و 6 أسابيع. من

كل مجموعة زراعة ، تم تقييم (8) غرسات من خلال اختبار عزم الإزالة و (2) زرع من خلال الملاحظات النسيجية من كل فترات الشفاء.

**النتائج:**

تقنية الغمر لزرعات التيتانيوم النقيه تجاريا المطليه بكبريتات الكالسيوم النانويه سوف تحسن صلادة السطح لطبقة الطلاء ,من الناحيه النسيجيه فان الزرعات المطليه بكبريتات الكالسوم النانويه والمستخدمه مع مسحوق قشور البيض كمادة تطعيم عظميه حول هذه الزرعات اظهرت تحسين وزيادة مستوى الاندماج العظمي بشكل افضل من المجموعات الاخرى , بينما القياس النسيجي و مكانيكيا (اختبار عزم الازاله) بعد اسبوعين وستة اسابيع من الزرع اظهر زياده محسوسه بالمقارنه مع المجاميع الاخرى.

**الاستنتاجات :**

يمكن أن تعمل كبريتات الكالسيوم النانويه بشكل ناجح كمواد طلاء لزرع التيتانيوم بواسطة طريقة غمر والتي تزيد من صلادة السطح وتمتاز بسماكة موحدة لطبقة الطلاء وتعمل بشكل كبير مع مسحوق قشور البيض كمواد طعوم عظمية حول الزرعات حيث تزداد كمية منطقة تكوين العظام الجديدة بالمقارنه مع المجموعات الأخرى.

**جمهورية العراق**
**وزارة التعليم العالي والبحث العلمي**
**جامعة بغداد**
**كلية طب الأسنان**

# دراسه استعمال كاربونات الكالسيوم المشتقه من قشور البيض كمادة تطعيم للعظم حول زرعات الاسنان المطليه بكبريتات الكالسيوم النانونيه

مقدمة الى مجلس كلية طب الاسنان/ جامعة بغداد كجزء من
متطلبات نيل درجة الماجستير في صناعة الاسنان

من قبل
**ذر رياض كاظم**
بكالوريوس طب وجراحة الفم والاسنان

إشراف
**أ. د. ذكرى اسماعيل حمد**
B.D.S., M.Sc., Ph.D.

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