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Enhancement of Early Osseointgration by Coating TiO_2 Nanotubes with Annealed Fishbone(In Vivo Study)

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Abstract
New method was used to accelerate bone osseointegration, milled and annealed fishbone was used as coating layer on Ti-6Al-4V alloy after creating TiO_2nanotube(TNTs) on it. Mechanical and thermal treatments were used to extract natural Hydroxyapatite (HAp) from fishbone.After milling, fish bones were heated at different temperatures. Annealed fish bones at 900^0C had correspondence structure to that of standard one as X-ray diffraction (XRD) confirmed. After creation TNTs on screws which were made from Ti-6Al-4V alloy, EPD was used to coat them with milled fishbone, annealed fishbone and commercial HAp. All screws were implanted inside the tibia of white New Zealand rabbits to evaluate the biocompatibility of modified alloys and to assess the clinical success of implants. Radiographic and histologic evaluations showed that implants with double surface modifications illustrate new bone formation around them. These results refer to the success of all surface modifications in this work , however the comparison between them illustrate superiority of coating by annealed fishbone over the other coating. During implantation interval from 4 to 12 weeks, screws with double surface modifications (creating TNTs and then coating with annealed fishbone) had highest removal torque (RTQ) values i.e. highest osseointegration acceleration rate. After 12 weeks of implantation, torque values for screws coated with commercial HAp are approximately equal to those values associated with screws coated with annealed fishbone.
Keywords:Ossseointegration,Titaniumimplants,Hydroxyapatite, TiO_2Nanotube,Fishbone.

1.Introduction

The feature that distinguishes bone from other connective tissue is the mineralization. The mineral is calcium phosphate (CaP), in the form of Hydroxyapatite (HAp) crystals $[Ca_{10}(PO_4)_6(OH)_2]$.HApwas first used in 1985 during clinical trials which showed increased osseointegration after the new implants began interacting with the bone after only 10 day post insertions. Since that time, HAp has been widely studiedand used in many in-vivo studies using animal models to examine the efficiency of HAp coated devices for enhanced stability and interaction with the natural bone tissue [Yanget.al.2005].
There is no adverse response to the material when HAp is implanted, since it is a substance naturally produced by the body. The idea behind coating implants with HAp would be for the body to chemically recognize the implant as bone and build living bone tissue into the coating. So HAp is very biocompatible **[** Sun et.al.2001**].**The use of HAp-coated implants has been reported to stimulate bone healing. This has been attributed to HAp'sosteoconductive property, thus resulting in an improvement in the rate and strength of initial implant integration **[**Ong et.al.2006**].**
In addition to animal models, documented medical reviews have reported that in both total hip and total knee arthroplasties in humans, the use of HAp coating has been shown to increase the interaction and osseointegration of bio inert metal implants **[**Epinette et.al.2004**].**
There are two types of apatite available, naturalapatite and synthetic apatite. Natural apatites are the ones prepared from animal bones such as coral and fish bone **[**Jensen et.al.1996**].** Synthetic apatite is prepared by chemically reacting a hydroxide source with a phosphate source.Commercially HAp powder is very costly, so an attempt is made to prepare the powder from animal bone (e.g. fish bone) as these sources consider as waste material. These are available and they are free of cost. Fish bone material becomes an important source for biomedical applications due to the presence of HAp as themajor inorganic constituent. Attempts have been taken to isolate fish bone derived HAp and use them as an alternate for synthetic HAp**[**Ozawaet.al.2002**].**
The production of synthetic HAp needs very complicated and sophisticated technique during synthesizing process. Whereas extraction of HAp from the natural bone is biologically safe and economic since this bone is easy to obtained. HAp produced from natural bone inherits most properties of the origin bone such as its chemical composition and structures give advantage in surgical applications. Besides, its potential for bone grafting is better than synthetic HAp **[**Hiller et.al.2003**].**In the best of our knowledge, there is no in vivo study investigate coating TNTs with milled and annealed fishbone. So, the aim of this contribution is investigation the

effect of coating TNTs with milled and annealed fishbone on the early acceleration of bone osseointegration.

2.MATERIAL AND METHODS2.1 Preparation of Medical screws
Screws was machined from Ti-6Al-4V alloy rod using wire cut machine, the length of the screw was 8mm. All screw body was threaded; the head diameter was 3.5mm while body was 3mm in diameter. They have a slide in the head of 1.5mm depth and 1mm width.**2.2 Anodic process of as machined Ti-6Al-4V surgical screws**Anodic cell consists of graphite cylinder act as cathode and screw of titanium alloy as anode. The chemical solution of the anodic process consists of NH_4F:H_2O: glycerol at a ratio of 1:20:79 wt. %**[**Park et.al.2010**]**. **2.3 Extracting HAp from the bones of Tigris river fish2.3-1 Fishbone cleaning process**The natural raw material used as a source of HAp was obtained from Iraqi fish bones (type fish cat) according to the following procedure. The fish bones were boiled in water for 1 h and washed using a strong water jet to eliminate the fish meat. The first milling step was done by using food mixer, then, hot air jet was used to dehydrate the bones. **2.3-2 Milling process**

The second milling process was done by using milling machine to obtain fine fishbone.The milling duration by this machine was eight hours; the number of milling container rotation was 200 cycle/minute. Milled fishbone particle size was measured by using laser diffraction particle analyzer, type Sald-2101-SHIMADZU,distribution of them is drawn in Figure 1.Statistical parameter of fishbone particle diameter distribution was recorded in table 1.

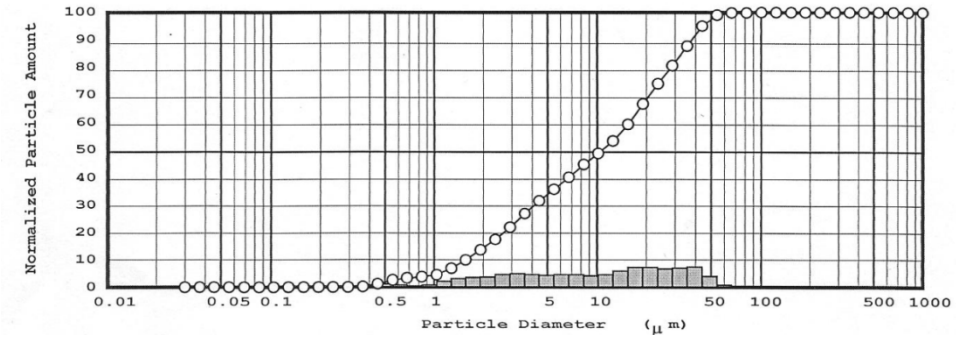

Figure 1 the distribution of fishbone particle diameters after milling process.

Table 1 some statistical parameters of milled fishbone.

Median(μm)	Mean(μm)	Standard deviation
10.752	8.666	0.534

2.3-3Fishbone heat treatment
Annealing of fishbone particles were carried out after milling process by using furnace [Hermann- Moritz, Italy]. Heat treatment was done under inert gas (argon) during two hours at different temperatures (500,800 and 900^0C).
2.4 Chemical deposition of milled and annealed fishbone on anodized Ti-6Al-4V alloy discs and anodized Ti-6Al-4V surgical screws Little amount of Poly vinyl alcohol (PVA) was dissolved in hot water, and then added to a mixture of milled fishbone (or annealed fishbone) and artificial ethanol. The final mixture then stayed under stirrering for 24 hour. After that, the final mixture was put in the same anodic cell. The voltage difference between cathode (graphite) and anode (Ti-alloy disc or screw) was maintained at 120 V for three minute. During this chemical deposition, the mixture solution was kept with continuous stirrering. PolishedTi-6Al-4V alloy and coated one with annealed fishbone is shown in Figure 2.

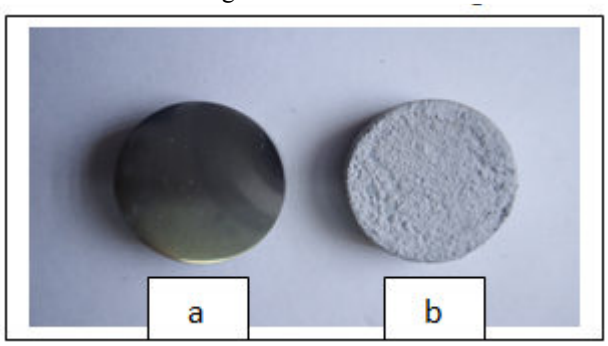

Figure 2. a :Untreated Ti-6Al-4V alloy. b:Ti-6Al-4V alloy after double surface modification: creating TNTs and then coating with Annealed fishbone at 900^0C.

2.5- Electrophoretic deposition of commercial HAp

Commercial HAp [from E.MERCK Darmstadt Germany] was deposited on screw that already had TNTs on its surface. The screw was put on the cathode electrode and a piece of the pure Ti was used as an anode electrode. Both electrodes were connected to the power supply and the ammeter. The distance between the electrodes was 10mm.The conditions of electrophoretic deposition process were tabulated in table 2. .

Table 2 electrophoretic deposition conditions of commercial Hydroxyapatite.

Material	Additive	Solvent	Temperature^{0}C
HAp	2.2g/l PVB	Ethyl alcohol	20

2.6 Characterization studies

2.6-1 Phase analysis studies

The crystalline nature of the materials, which were used in this study was tested by powders X-ray Diffractometer using Cu Kα radiation.

2.6-2 Scanning Electron Microscopy To study the changes occurs on the surface during the anodizing process the Anodized surfaces were examined using Scanning Electron Microscopy SEM (JEOL-JSM-5600). Samples for SEM were prepared as cross-section to find out the layer of the modified surface.

2.7 Surgical implantations

Forty two adult male New Zealand white rabbits, aged 8-12 months, weighed 1.5-2 kg were used in this study. They were left for 2 weeks in the same environment before surgical operation. The total animals were divided into five groups for each healing interval (2, 4, 6,12 and 18 weeks) each one consist of 9 animals except last period consists of 6 only as it subjected to mechanical test only. Three of each group was sacrificed for histological study, while the other six had been tested for mechanical evaluation by (RTQ) test. Five implants (Figure 3) were implanted in the tibiae, three implants (uncoated, with TNTs+ milled fishbone and TNTs + commercial powder HAp) were implanted in the right tibia and the other two (TNTs only and TNTs + Annealed fishbone at 900^{0}C) were implanted in the left tibia. Before operation the required dose of anesthesia and antibiotic was calculated by weighting each rabbit in a special balance for the animals.

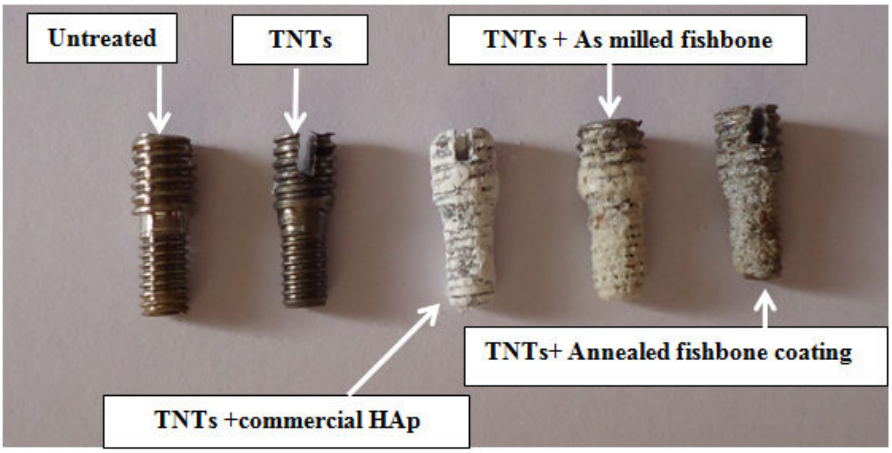

Figure 3 surgical screws with different surface modifications.

Anesthesia was induced by intramuscular injection of ketamine hydrochloride 50 mg (1ml/kg body weight) and xylocaine 10% (1ml/ kg body weight).All instruments and towels were autoclaved at 100 ºC for 30 minutes.Both tibiae were shaved with shaving cream from the inner side and then the skin was cleaned with ethanol. Surgery was performed under sterile condition and a gentle surgical technique.

2.8 Implant Recovery and Mechanical Testing

The animals that categorized for mechanical test were anesthetized with the same type and dose that used in the implantation procedure. The implant stability was checked by measurement of the mobility of the implant using two hand instruments after supporting of the tibia from the inner side. A (RTQ) test was performed by engaging the screw driver of the torque meter into the slit in the head of the implant to measure the force (peak torque) necessary to unscrew the implant from its bed**.** Figure 4 shows radiograph of the implant screws after surgery. This image illustrates that the bone of the tibia was sufficient to fit the diameter and length of the implants and the implants were properly inserted in their positions.Muscles and fascia were reflected to expose the implants,see Figure 5.

2.9-Histological evaluation

Twelve animals were scarified for histological evaluation under light microscope .The animals killed by overdose of ansthesia. The following steps for preparing the bone-implant bock including:

1. Cutting of the bone around the implant was performed using a disk in low rotating speed with normal

saline cooling**.** Cutting was made about 5 mm away from the screw.

2. Bone-implant blocks were immediately stored in 10% freshly prepared formalin and left for 72 hours for fixation**.**
3. Decalcification ,washing.dehydration, embedding in paraffin wax and sectioning ,staining by H&E stain then evaluated under light microscope **[**Hammad.2007**]**.

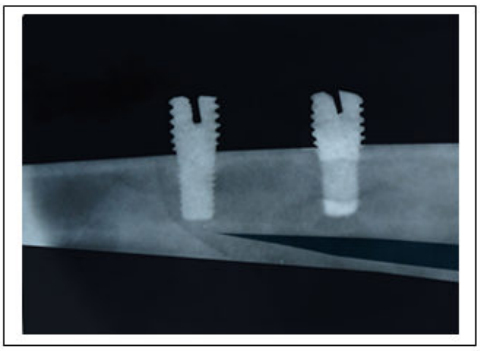

Figure 4 Radiograph of the implant after one month surgery.

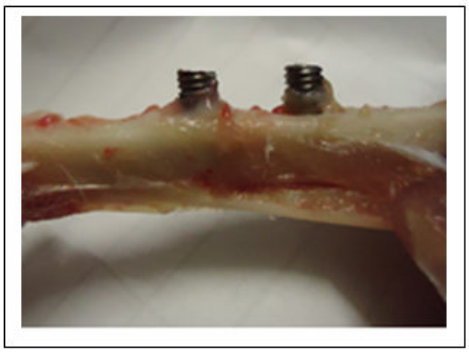

Figure 5 Side view of the exposed tibia with screws in position.

3.RESULTS AND DISCUSSION

3.1 Morphology of TNTs for anodized Surgical Screws and their EDS spectra.

SEM image and corresponding EDS spectra for anodized screw are illustrated in Figure (6-A,B). It's clear that the TNTs are not uniform or have the same length over whole surface. It's well known thatthe arrays of nanotubes formed on α phase region while the selective dissolution of β phase occurs, leading an inhomogeneous oxide nanotube layer[Stoica et.al.2012].

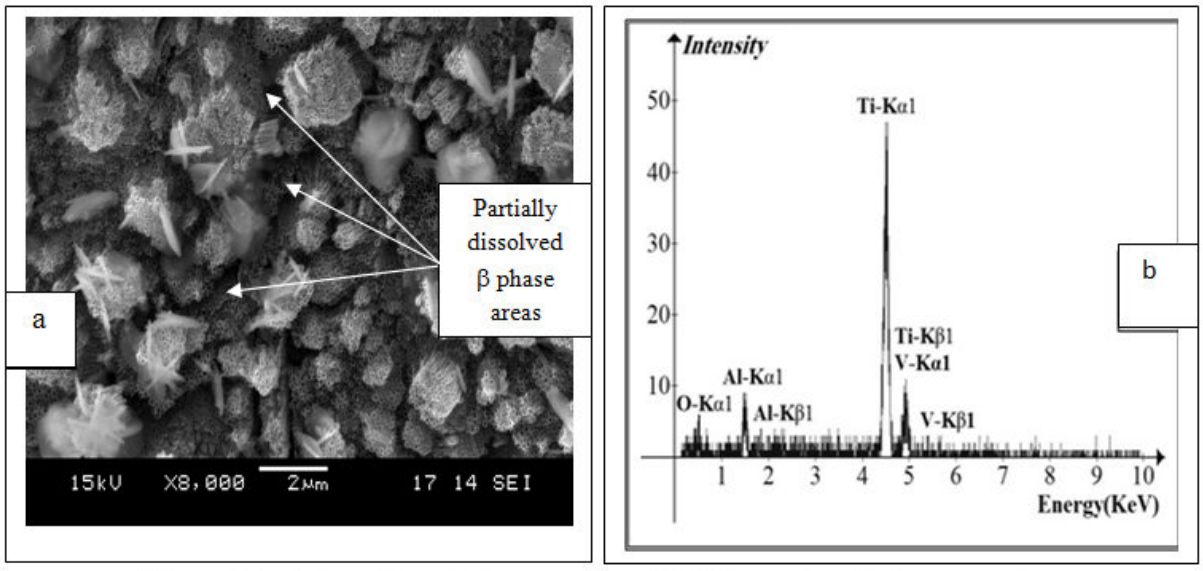

Figure 6 Morphology of anodized screw surface (a) TNTs spread on all the screw surface. (b) is EDS spectra of corresponding images.

3.2 Natural HAp phase analysis

Figure 7shows the XRD patterns of untreated and heated fishbone at various temperatures. The data show that the XRD pattern of annealed fishbone at 900^0C matches well with the XRD pattern of standard HAp, confirming that the material derived from catfish bones was indeed HAp. Untreated fishbone XRD shows broad peaks and indicates to the amorphous nature i.e. low crystallinity of HAp. Under heat treatment from 500 to 900°C, the bone gradually transformed to a well crystallized HAp, minimal line broadening, high intensities and hard peaks sharpness. The appearance of tricalcium phosphate $Ca_3(PO_4)_2$ phase in the sample annealed at 900 °C indicates the decomposition of HAp **[**Prabakaran et.al.2006**]**, Thus, it is possible to develop phase pure HAp at temperature equal to 900 ^{0}C. In general, XRD patterns show that the stoichiometric HAp remains stable without the formation of decomposition products at annealing temperatures 500 and 800 ^{0}C. There were no other calcium

phosphate phases to be traced.The black powder, produced by annealing process, began recrystallization at about 500 ^{0}C without decomposing to any other compound of the calcium phosphate family.

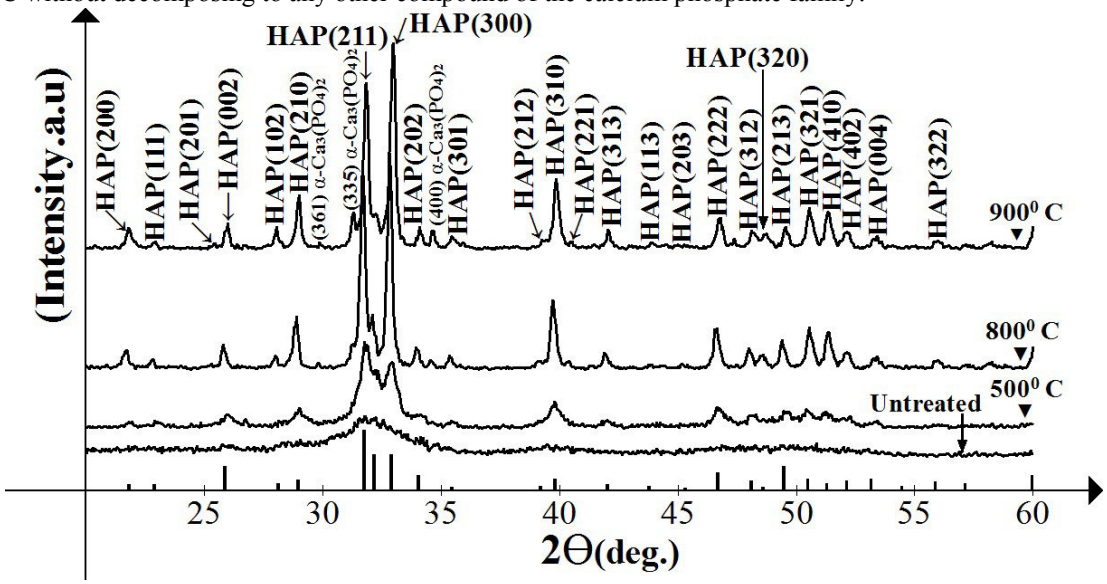

Figure 7 XRD for untreated and annealed fishbone at different temperatures.

3.3Radiographic evaluations

There were no areas of radiolucency between the implant and adjacent cortical bone in any specimen of radiographic examination corresponding to clinical observation for no gross change in the tibial architecture noted in all specimens .

Figure 8 shows apposition of newly thick bone around all surface modification in comparison to control.

3.4 Mechanical and histologic findings

The strength of bone implant integration was evaluated by (RTQ) test which measures the torque (force) needed to loosen the implant in the bone bed. This is a 3-dimensional biomechanical measurement roughly reflecting the interfacial shear strength between bone and implant. Figure 9illustrates the following results:

I. In all cases, (RTQ) increased with increasing implantation interval.

II. In the interval from 2 to 4 weeks TNTs implants record high torque value. While at 6-12 weeks screws with double surface modifications (creating TNTs + coating with annealed fishbone) record the highest value. This high(RTQ) refers to good osseointegration as confirmedby **[**Patricia. 2006**]**

III. After 12 weeks of implantation, torque value unchanged and stilled approximately at steady state for all surface modifications (except values associated with screws coated with milled fishbone).

IV. After 12 weeks of implantation, torque values for screws coated with commercial HAp are approximately equal to those values associated with screws coated with annealed fishbone.

The present study likes other researches illustrates that; histologic findings confirm and support RTQ results. These confirmations are:

A-RTQ values are good indication to evaluate bond strength between implant and bone **[** Johansson and Albrektsson , 1987**].**

B- Increasing implantation interval produces strong bond as a result to bone remodel. The fast bone healing gives high RTQ values **[** Krautet.al.1991**].**

C- Increasing RTQ values related to transformation of woven bone to mature one. Since the woven bone mostly consists of irregular shaped and loosely packed collagen fibers,that it does not provide sufficient mechanical stability compared to the organized the lamellar bone **[**Chappardet.al.1999**].**

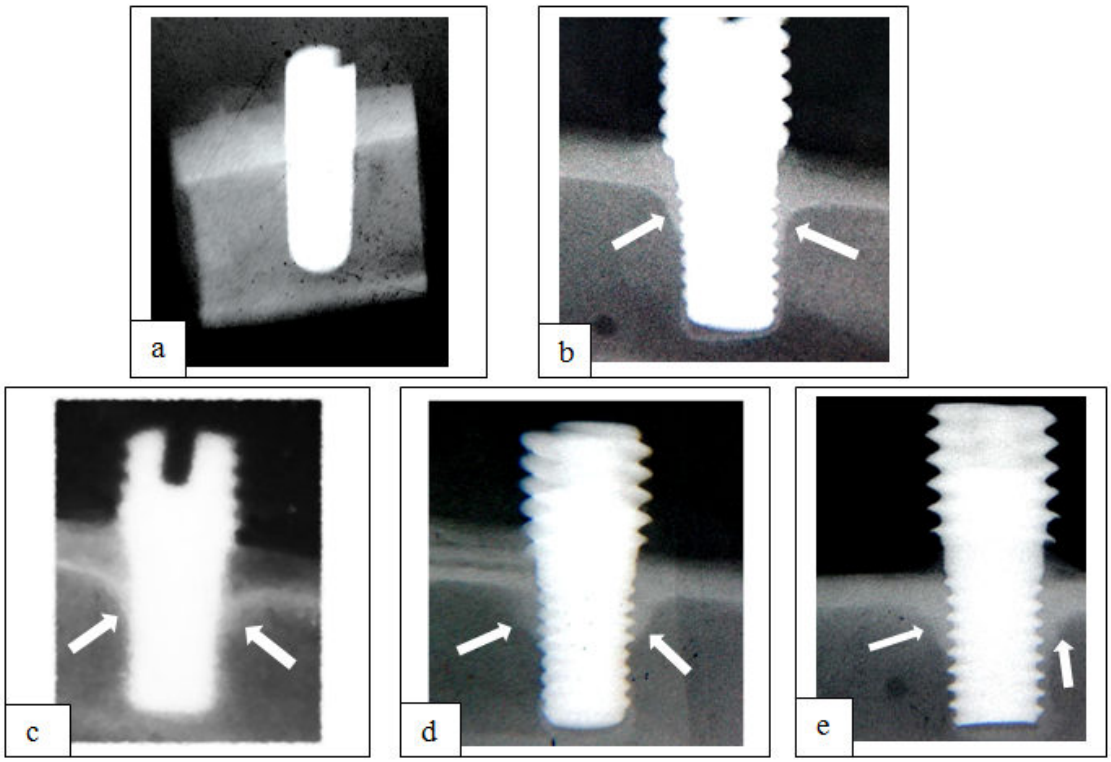

Figure 8 Radiograph of new bone around screws with different surface modifications.a-Untreated implant. b-Double surface modification (creating TNTs then coating with milled fishbone).c-Double surface modification (creating TNTs then coating with commercial HAp).d-Double surface modification (creating TNTs then coating with annealed fishbone).e- Only creating TNTs.

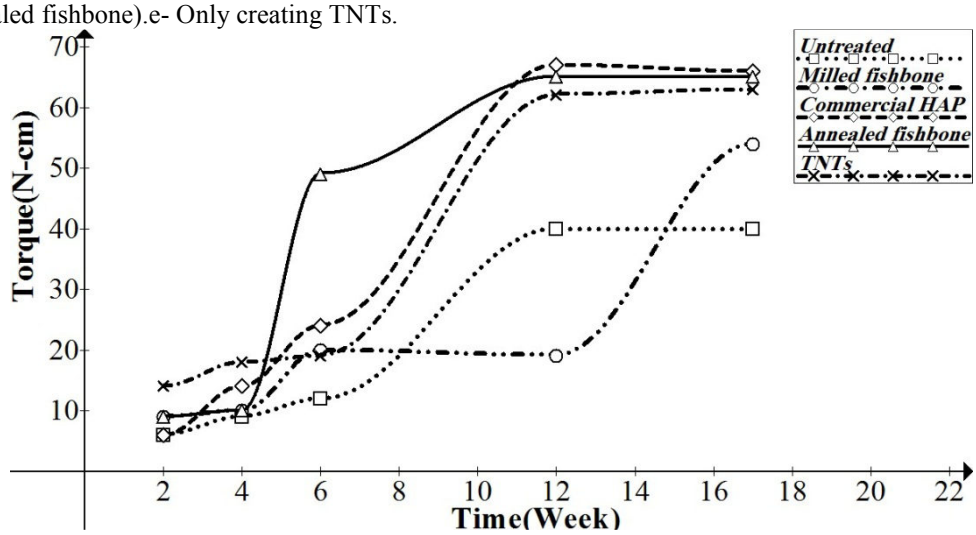

Figure9 (RTQ)as a function of implantation interval.(***Note: each point in any curve represent the average of six torque meter test reading)***

D-The ability of untreated implant to activate osseointegration is limited.

E- Dissociation rate of Ca and P elements from HAp coating plays important role in healing process.

F- Partial dissociation of HAp coating increases the concentrations of Ca and P elements in fluid surrounding screw, leading to motivate construction of new bone. Dissociation of Ca and P triggered the progenitor and formative bone cells to appose bone**[**Almeida.et.al.2005**].**

G- Rough surfaces have better effect on osseointegration **[**Wennerberg,2003**].**

H- RTQ values directly proportional to surface area of contact between implant and bone**[**Ivanoff et.al.1996**]**.

Implant coated with milled fishboneshows that increasing RTQ values stopped for 6 weeks then they increased. This behavior may be attributed to amorphous structure of this coating as confirmed by (XRD) pattern.The same reason may be explains the lower RTQ values for this implant compare with those for implants coated with annealed fishbone or commercial HAp coating. Besides, for this implant, histologic image in Figure10

illustrates spread mature bone in some contact area and spread bone marrow in the rest. The result of this incomplete transformation to mature bone is low RTQ value for this implant. Bone rebuilding has a direct relation with calcium and phosphor dissociation from HAp.Wenisch[Wenisch et al.2003]showed by ultra-structural investigation in female sheep using transmission electron microscopy that osteoclasts contributes to degradation of calcium phosphate ceramics by means of resorption and phagocytosis. So, for milled fishbone it may divide this dissociation process to three stages during 18 weeks: Active dissociation process during first 6 weeks, inactive dissociation process in-between 6 and 12 weeks (RTQ values were constant) and active dissociation process during12 to 18 weeks (increasing RTQ values to relatively high values).

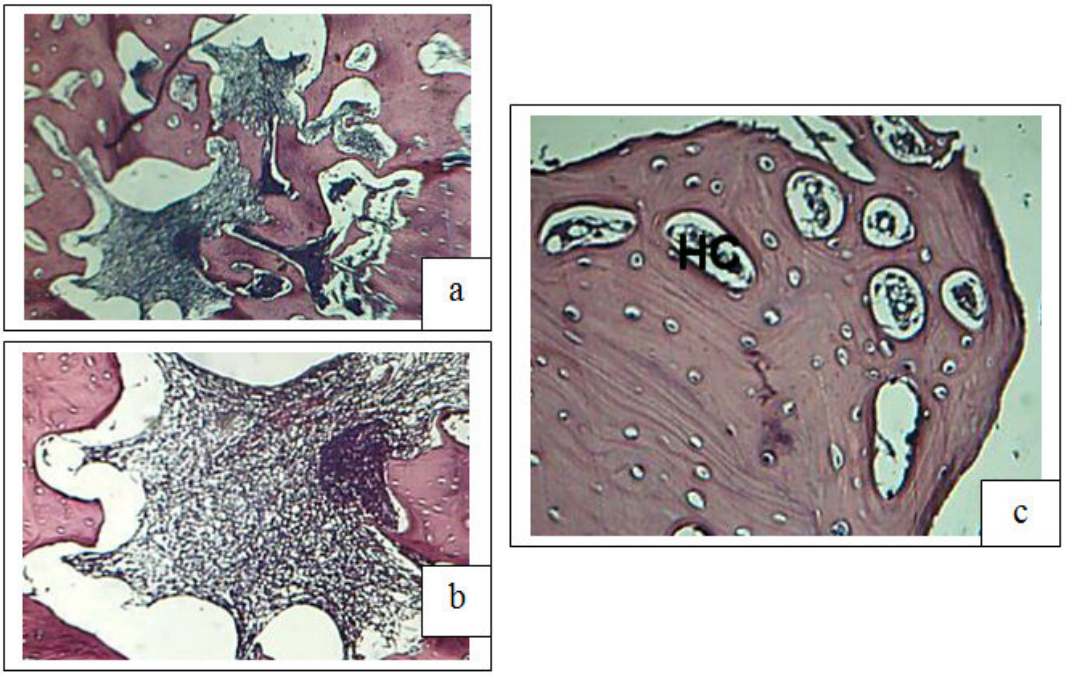

Figure10 a:View for new bone filled the interface zone of milled fishbone group (12 weeks).H&EX4. b:View for thread interdigitated bone marrow in interface zone.H&E X 10. c:Magnifying view for 'a' shows havarsian canal(HC).H&E X 20.

There were two surface modifications that produced complete transformation from immature to mature bone during 12 weeks.

The first one is implants covered by TNTs only.Formation TNTs on the implant surfaces before coating results in high RTQ values for implants coated with commercial HAp or annealed fishbone. TNTs facilitate blood flow through them and then increasing interaction between coating and implant. Also, TNTs increase surface roughness which is necessary for good osseointegration. These tubes give larger contact area between implant and coating material result in high RTQ values. The original implant area was increased due to forming TNTs, which in turn resulted large OH^- absorption ability and then increasing the numbers of nucleation centers to deposited HAp. Figure 11 shows haviersian canal inside bone structure which touches implant (creating TNTs only). This bone structure indicates to advance step of bone osseointegration .This result agree with many authors[Steinemann.1996, Skriptiz et.al.1998].Two mechanisms have been proposed to explain the osseointegration around this implant : mechanical interlocking through bone growth in pores, and biochemical bonding[Sul et.al.2005].TiO_2 appears to play a central role in the osseointegration of titanium-based implants, and direct chemical bonding between the bone tissue and TiO_2 has been postulated[LeGeros et.al.1993].Also several in vitro studies have demonstrated that cells cultured on the nanotubular surfaces showed higher adhesion and bone matrix deposition [Popat et.al.2007].

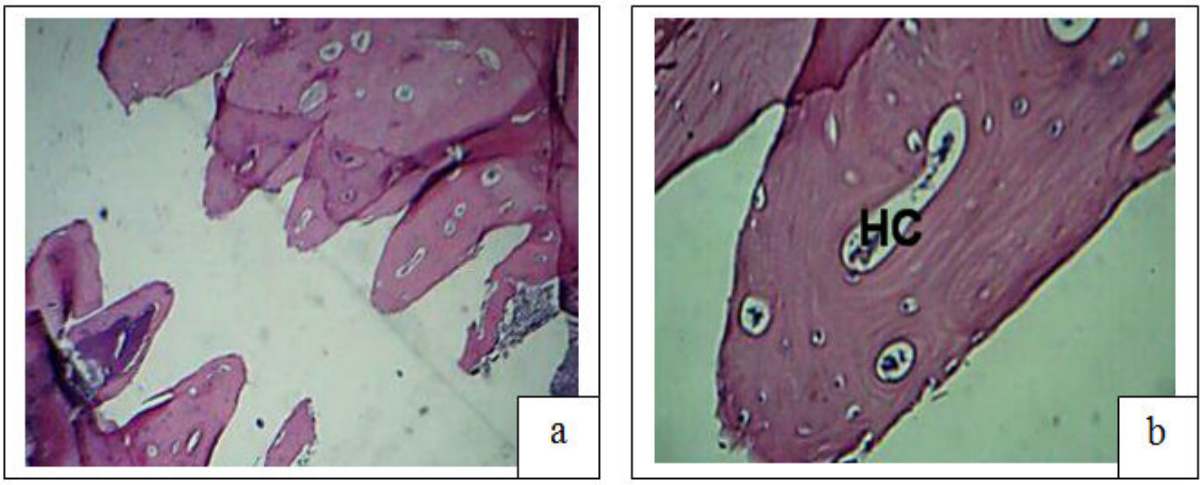

Figure 11 a:Boney threads of implant,nanotube group (12 weeks).H&E X 40.
b:Magnifying view for the thread in 'a' shows haviersian canal(HC).H&E X 20.

The second surface modification produced mature bone after 12 weeks from surgical treatment was coating the screw with annealed fishbone, as Figure12shows. The reasons behind this success may be one or all the following:

●- Creation of porous fishbone due to organic materials evaporation. Porosity is an important factor because it increases the interaction surface area between the blood and fishbone **[**Shepherd et.al.2011**] .**

●-Annealed fishbone at 900^0C had high crystalline structure.This structure may have a good action on acceleration of ossiointegration, because some studies**[**Suzanne et.al.2008**]** indicated higher bone activity with highly crystalline films.

●- X-Ray Fluorescenceresults illustrate that, increasing Ca and P concentrations after annealing increase the probability of usage these elements in ossiointegrationprocess, see Table 3 .

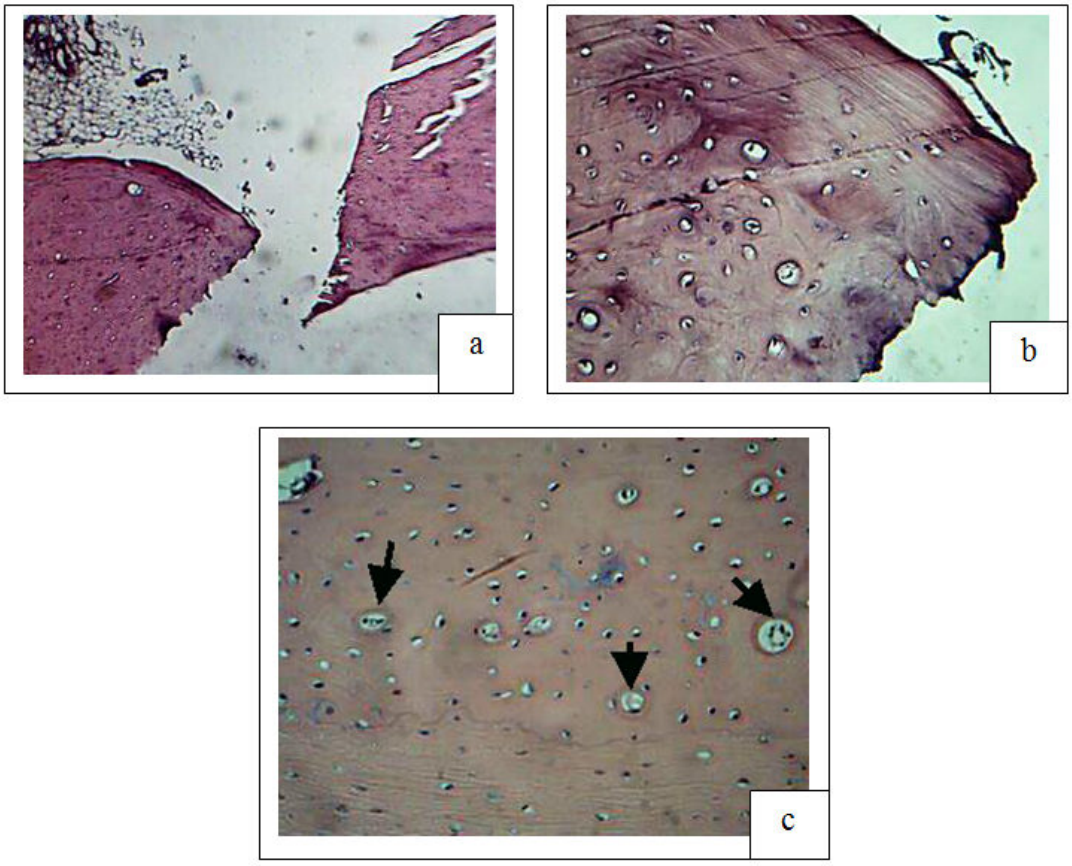

Figure11 a:View for thick mature threads in annealed group(12 weeks). H&E X 10. b:Magnifying view for mature bone in previous Figurea . H&E X 20. c:Other Figure for mature bone with multiple haversian

canals(arrow). H&E X 20.

Table (3) Inorganic materials concentrations(exceed 0.1%) of fishbone before and after annealing.

Element	Concentration before annealing	Concentration after annealing
Magnesium	0.388%	1.031%
Phosphorus	8.943%	16.77%
Calcium	22.01%	39.95%

Conclusion

1. Extracted Hydroxyapatite from Iraqi fishbone has excellent correspondence with standard after sintering at 900^0C.
2. Electrophoretic deposition is successful method to coat medical screws with milled and annealed fishbone.
3. The present study also indicated that the Ti-6Al-4V alloy and the coated materials (milled and annealed fishbone) are tolerated by body and recorded to be inert as revealed by the lack of adverse tissue reactions, advanced healing and the high level of (RTQ)value records.
4. Creating Double surface modifications (creating TNTs+ coating with annealed fishbone) is active method to accelerate early stability of implants and enhaced osseointegration.
5. At 12 week postoperatively,mature bone is recognized in two types of implants: Fist one with double surface modifications (creating TNTs + coating with annealed fishbone) ,the second which was covered by TNTs only.

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