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In Vivo Study of Hydroxyapatite-Chitosan and Hydroxyapatite-Tricalcium Phosphate Bone Graft in Sheep's Bone as Animal Model

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Abstract – This study describes the in vivo evaluation study of bones implanted with graft of hydoxyapatite-chitosan (HA-C) and hydroxyapatite-tricalcium phosphate (HA-TCP) composite in sheep's bone as the animal model for human. This study was done in two parts, non-invasive clinical imaging study, i.e. brightness mode ultrasound (B-mode US) and conventional radiography (CR) technique, and morphological study of the bone healing process. Six sheep were used in this study and separated in two groups, three sheep for each group. The implantation surgery was done aseptically by creating a 4 mm diameter and 7 mm depth hole on each left and right hind limb of proximal medial tibial bone. First group implanted of HA-TCP at the left tibial and the second group by the HA-C composite. The right tibial of both groups served as control, which was drilled but not implanted. The CR and US were performed one day before and 7, 21, 30 days after implantation. Bones were harvested after 30, 60, and 90 days post-surgery and observed for morphological study. The B-mode US showed that inflammation and early bone remodeling occurred of both implants at subcutaneus area seven days after implantation and decreased 30 days after. Furthermore, the CR technique showed that both implant were still intact on site 90 days after, therefore, indicated minimal absorption or even not absorbed at all. The morphological evaluation showed that HA-TCP had been degradable 30 days after and continued, indicated signs for biocompatibility, biodegradability, bioresorbable, bioactivity and osteo conductivity properties. On the other hand, HA-C graft showed only biocompatibility characteristics 90 days after implantation. The result showed that the healing process of HA-TCP faster than HA-C but nevertheless, both composite still slower healing process than control bone.

Keywords: hydoxyapatite, tricalcium phosphate, chitosan, bones implant, in vivo.

I. Introduction

Every year million people suffering of bone disease caused by accident, cancer, or fracture.[1] In Indonesia, some of the bone cancer, periodontics, traumatic, fracture of bone still increased every year.[2] One of the most popular to treatment this problem are use implantation technique to remove and replace broken bone or disappear from bone. In other words, demand of bone graft or biomaterial that used as bone at the same time increases.[3]

Biomaterials are natural or synthetic material that used at biological system. This material aim to repair, restore, replace broken tissues as the interface with the physiological condition.[2] Synthetic biomaterial must have ideal properties like bioactive, biodegradable, bioresorbable, and biocompatible with the body tissue for long time.[4] Ideal materials to replace bone must have some properties like a osteoconductive, steoinductive and integrated to bone structure.[5]

The synthetic material used as a bone graft must be bio-compatible and bio-active. Apatite is a bone composer material. The main component of apatite compound is calcium phosphate, which one of the phases is hydroxyapatite (HA) which posses the most stable form. To match it, TCP (tricalcium phosphate) which has a higher absorbance is added. The HA-TCP combination is expected to be used in bone implantation. Chitosan (C) is a form of natural hydroxyapatite that can be found in the nature and has a good osteoconduction and bio-compatibility inside the tissue.[6]

Currently, non invasive clinical examination of bone disease or trauma (fracture, fissure etc) is still using ionizing radiation energy such as radiography, CT scan, and fluoroscopy.[7,8] In the other hand, B-mode US device may prove to be more effective in assessing bone micro-architecture, the onset of bone formation, and the surface topography of bone.[9,10,11] However, CR is not a perfect diagnostic or monitoring tool, because a soft tissue healing may not be obvious and because

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the extent of lesion and spatial relationship to important anatomical landmarks are not easily visualized.[12]

Morphological study of bone healing process and disease can be performed by macroscopic (pathological anatomy) and microscopic (histopathology) examination. Pathological anatomy is the diagnosis of disease based on the gross examination of organs, tissues, and whole bodies. Histopathology refers to the examination of a biopsy or surgical specimen after the specimen has been processed, and histological sections have been placed onto glass slides and stained with general stain such as Hematoxylin-Eosin (HE) or specific stain such as Von Kossa's-calcium method. Histopathology examination of bone can describe the types of cells (osteoblast, osteocyte, osteoclast, etc.), the proportion of cells to the matrix, and the properties of the matrix itself.[13]

This research was aimed to evaluate in vivo study of bones implanted with graft of hydroxyapatite-chitosan (HA-C) and hydroxyapatite-tricalcium phosphate (HA-TCP) composite in sheep's bone as the animal model for human. This study was divided in two parts, non-invasive clinical imaging study, i.e. brightness mode ultrasound (B-mode US) and conventional radiography (CR) technique, and morphological study of the bone healing process.

II. Material and Method

Bone Implant Synthesized

Hydroxyapatite-tricalcium phosphate (HA-TCP) composite. HA-TCP was synthesized by reacting with 100 ml CaCl2.2H2O solution with 100 ml Na2HPO4.2H2O using the precipitation method and then formed into a pellet.

Hydroxyapatite-chitosan (HA-C) composite. HA-C was synthesized using the precipitation method and then formed into a pellet. HA-C implants from the precipitation method has a 0.5 M: 0.3 M Ca/P ratio. HA-C synthesis was done in two steps, HA forming and HA mixing with C.

Animal Implantation

Six local sheep were used in this study ranged from 1-1.5 years old, weighed 15-20 kg. All of sheep were divided into two groups, HA-TCP and HA-C that consisted of three sheep for each group. The implantation surgery was done aseptically by creating a 4 mm diameter and 7 mm depth hole on the left medial on diafise tibial bone. The surgery was performed after anaesthetized by 0.1-0.22 mg.kg-1 Xylazine 2% i.m. injection. Premedication with atropine sulfas 0.2 mg.kg-1 were injected 10 minutes after Xylazine injection.[14] The sheep were received bone implant at the left proximal medial tibial by HA-TCP or HA-C at the difference group. The right tibial bones were drilled as well as left with no implantation served as a control group.

Ultrasound Examination

Bone implants were examined using a portable ultrasound machine (KX 5100, Xuzhou Kaixin Electronic Instrument Co., Ltd, China) with a multi frequency linear transducer (5-7.5 MHz) and a thermal printer. The US performed before, 7, 14, 21 and 30 days after implantation. US imaging for longitudinal and transverse views from the dorsal and the distal aspect of the implants were taken. Parameter of evaluation consisted of size of site, implants, new bone formation, periosteal soft tissue swelling and surrounding to implant site soft tissues were evaluated.[15]

Radiographic Examination

Bone implants were examined using a portable x-ray machine (VR-1020, MA Medical Corp. Japan). The CR was performed one day before and 7, 21, 30 days after implantation. Standard radiographs of tibial bones were obtained in caudo-cranial and latero-medial Kilo-Volt peak (kVp) and milli ampere second (mAs) arranged that they can produce the best quality of radiographs with the focal-film distance (FFD) 80 cm for each sampling data. Film washed in the dark room and then dried. The radiographs evaluated in front of an illuminator panel. Evaluation parameter was consisted of margination, opacity, implant size, shape, dimension, new bone formation, periosteal soft tissue swelling and surrounding to implant site soft tissue.[15]

Morphological Examination

The samples were taken 30, 60, and 90days after implantation by cutting graft-contained bone and observed for macro morphological structure. The bone was processed using un-decalcification and decalcification method and stained with Haematoxylin-Eosin (HE) for micromorphological structure.

III. Result and Discussion

Clinical Non-invasive Imaging

US imaging evaluation: Sonogram of tibial bone before the drill showing the difference of skin echogenicity of skin, subcutaneus, bone and medulla of bone were present. The subcutaneus area (fig. 1ADG) at the 21 days after drilled (fig. 1D) were thicker than the undrilled region (fig. 1A) and consist of multi echoic images, hyper-, hypo- and an-echoic The hypo-echoic images were indicating of semi high reflective mass, like a primary callus. The hyper-echoic images were indicating of high reflective mass, like a secondary callus. Then an-echoic images were indicating of un-reflective mass, like a liquid. The drilled hole of tibial bone can be seen as an-echoic image. The hole diameter became a decrease after 30 days (fig. 1G), which indicate the bone remodeling were occurred.

Sonogram of tibial bone showing the HA-TCP implant site consist of hyper-echoic mass. The subcutaneus (sc)areas of tibial after 21-day implantations by HA-TCP were thicker than seven days and decrease after 30 days (fig.1BEH). Inflammation and bone remodeling were occurred after implantation that visible to sc areas. The inflammation of sc areas were thicker than normal undrilled bone and consist of multi echoic images. The hypo-echoic images were primary callus. The hyper-echoic images were indicating secondary

callus. Then an-echoic images were indicating the liquid. Same conditions were shown at the HA-C bone implants (fig.1-CFI).

Hypoechoic or anechoic structure was representing the soft tissue, which may extend around the bony contours.[16] As compared with CR evaluation of healing in fractured bones, US may prove to be more effective in assessing bone micro-architecture, the onset of bone formation, and the surface topography of bone.[9,10,11] Plain radiographs are the standard technique employed to document the typical bone remodeling and are, therefore, very useful.[17] However, they are insensitive to the soft-tissue changes that are the only signs of early inflammations. [18]

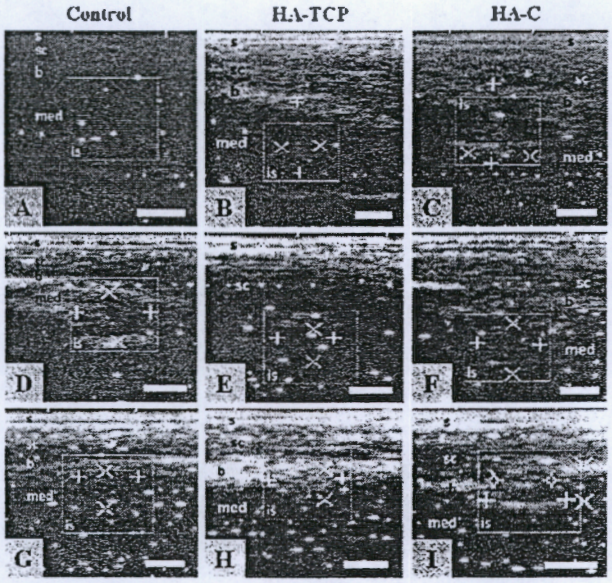

Fig. 1. Ultrasound (US) images of control, HA-TCP graft implanted, HA-C graft implanted tibial bone. A: Undrilled bone, D,G: Drilled bone at 21 and 30 days. B,E,H: HA-TCP implanted at 7, 21 and 30 days. C,F,I: HA-C implanted at 7, 14, and 30 days. s, skin; sc, subcutan; b, bone; med, medulla; is, implant site; arrow (→), anechoic image indicate that site consist of liquid, site of implant (□), size of hole site was showed by the distance between two "+" and two "x" mark. Bar = 10 mm.

Ultrasound (US) has multiple advantages: it is readily accessible, can be performed quickly without delay and with minimal discomfort to the patient, it is useful in regions that are complicated by orthopedic instrumentation and therefore, might not be well seen with CR, has a lower cost, does not use ionizing radiation, and offers real time imaging.[15,16]

CR imaging evalution: The HA-TCP and HA-C implanted bone still exist at the site until 90 days (fig. 2H, L). The defect at the control days 30 after implantation showed indistinct of margination and decreased to be disappear at 60 and 90 days after implantation (fig. 2B-D). HA-TCP and HA-C showed that bone implant more radiopaque than bone body texture. At the HA-TCP after 30 days, showed a radiolucent line at the implant site and become wider as radiolucent mass. Radiolucent mass was also found surrounding the implant site. This process still developed until 60 and 90 days after implantation (fig. 2E,F,G,H). in other hands, at the HA-C implanted condition until 90 days after implantation still intact, and no degradation process occurred (fig. 2.I,J,K,L).

From the CR imaging scale evaluation, we suggested that the HA-TCP bone implants have better properties for biodegradability and bioresorbsibility than HA-C bone implants. On the other hand, the process of bone growth which implanted by HA-TCP bone implant was slow. This condition may be caused by minimal osteoinductive properties of HA-TCP(fig. 3).

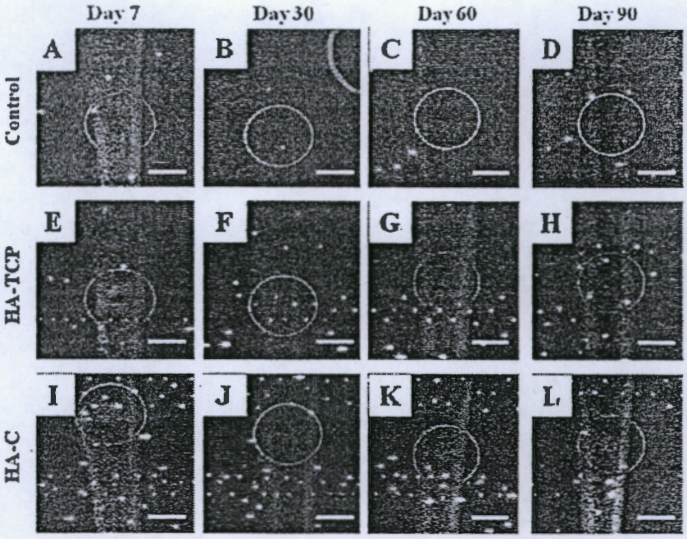

Fig. 2. Conventional radiography (CR) images of control, HA-TCP graft implanted, HA-C graft implanted tibial bone A,B,C,D. represented control group of 7, 30, 60 and 90 days after drilled. E,F,G,H. Represented HA-TCP group of 7, 30, 60 and 90 days after implanted. I,J,K,L. Represented HA-C group of 7, 30, 60 and 90 days after implanted. Bar = 5 mm.

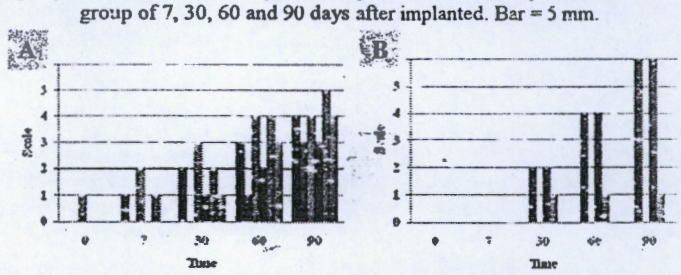

Fig. 3. Conventional radiographic scale evaluation of HA-TCP and HA-C graft before and 7 until 90 days after implantation. A. Radiograph evaluation of HA-TCP. B. Radiograph evaluation of HA-C. implant opacity (■), bone opacity (■), internal implant radiolucent image (■), peripheral radiolucent of the implant (■), margination (■), degradation size of implant (■). Scale 1 is describe the lowest level of bone transformation, scale 6 is the highest level.

Morphological Bone Implant

Macroscopic morphology: The macroscopic change of tibial bone and graft implant after implantations were shown at the table, I. From five parameters evaluation, clearly showed that HA-TCP graft implant has better characteristics than HA-C. HA-C grafts were still intact on implant site after 90-day implantations.

The HA-TCP implant has deteriorated and induced the connective tissue growth. Furthermore, those conditions was followed by the osteogenesis. (fig. 4A,E,I). The connective tissue was not coat the implant, instead it grows inside the implant. Degradation occurred at the HA-TCP implant. In other

conditions, HA-C showed the implant still intact and the connective tissue was not grown on the implant site (fig. 4C,G,K). on the other hand control group showed the defect has been closed with connective tissue or woven bone (fig. 4B, F, J and D,H,L).

TABLE I
MACROSCOPIC CHANGE OF TIBIAL BONE AND GRAFT IMPLANT AFTER IMPLANTATION[a]

No	Characteristic	Evaluation time (day)					
		HA-TCP implant			HA-C implant		
		30	60	90	30	60	90
1.	Implant condition	Deg	Deg	Deg	Ud	Ud	Ud
2.	Color	White	White	White	Ivory	Ivory	Ivory
3.	Form of implant	+ (crevice)	++ (crevice)	+++ (crevice)	Intact	Intact	Intact
4.	Degradation degree	+	++	+++	-	-	-
5.	Growth of new tissue at the internal implant	+	++	+++	-	-	-

[a]Undecalfification bone evaluation; (Deg) degradated , (Ud) undegradated, (-) none, (+) little, (++) enough, (+++) more

Physiologically, some of the hydroxyapatite compound has slow degradation and tricalcium phosphate absorbed after six weeks post-operative.[19] Ref [20] reported that TCP had the brittle characteristic. The defect at the control, filled by the bony callus or woven bone after 1-4 week and bone remodeling occurred 90 days.[21] In these steps, the bone had form, structure and mechanical properties like before.[22]

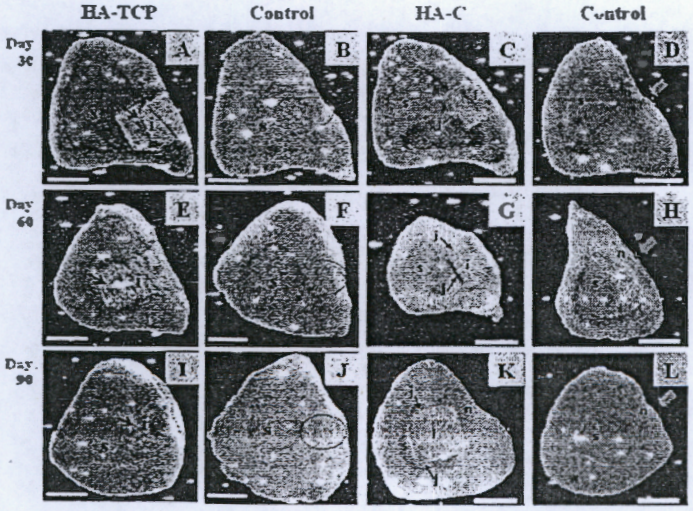

Fig. 4. Diagonal cutof control and graft-contained tibial bone. . A,E,I. Represented HA-TCP group. Degradation with cut in half like form of connective tissue (→) and to be deteriorate. C,G,K: Represented HA-C groupB,F,J and D,H,L. Represented control each groups. The (○ and →) sign location of bone defect. i = bone implant, j = connective tissue, n = woven bone, s = bone marrow. HE colored. Bar = 5 mm.

Microscopic evaluation

Microscopic morphology of undecalcification bone evaluation method: The bone formation as bone remodeling signs at the HA-TCP were clearly defined. The new bone tissue, proliferation of connective tissue, trabecular formation, biodegradation and neo-vasculars were found at the HA-TCP implant. On the other hand, HA-C groups were shown opposite results. The bone constructions were un-detected and the implant still intact at the implant area (table II).

The new bone formations were increasing until 90 days after implantation at the HA-TCP group. However, at the HA-C implanted group has the different results. Bone structures were constructed at the HA-TCP implant area. Trabecular form and woven bone were also growth on site (fig.5A,E,I). These conditions indicated that HA-TCP have osteo conductive properties for supporting the bone formation process. At the same time, the HA-C group has been showing the different direction sign of bone remodeling (fig. 5C,G,K). The new bone growth direction on the gap between implant, and old bones were described. The undegraded HA-C implants was became a barrier against bone growth.

TABLE II
MICROSCOPIC CHANGE OF BONE IMPLANT AFTER IMPLANTATION[b]

No	Characteristic	Evaluation time (day)					
		HA-TCP implant			HA-C implant		
		30	60	90	30	60	90
1.	Proliferation of connective tissue in to the internal implant	+	++	+++	-	-	-
2.	Growt of new bone tissue at pheripera! implant	-	++	+++	-	-	-
3.	Growt of new bone tissue at central implant	-	++	+++	-	-	-
4.	Bone marrow proliferation	-	-	-	-	-	-
5.	Connection old bone with implant	-	-		-	-	-
6.	Trabecular formating at internal implant	+	+	+	-	-	-
7.	Biodegradation	-	++	+++	-	-	-
8.	Inflamation reaction at pheriperal implant	-	-	-	-	-	-
9.	Neovascularisation at internal implant	-	++	+++	-	-	-

[b]Undecalfification bone evaluation; (-) none, (+) little, (++) enough, (+++) more

Ref [22] Kalfas (2001) reported, that osteo conductive properties should be the nature of the bone graft as a scaffold biomaterials. There will conduct of neovascularization and osteogenic cells infiltration to the graft. Therefore, HA-TCP is calcium based material who non-inflammation, non-irritating and non-immunological respon.[1] The ideal composition of composite has an important role of material properties.[23]

Currently, the ideal proportion of a biomaterial Chitosan based still in the research process.

Microscopic morphology of decalcification bone evaluation method: In the HA-TCP group, there was a proliferation of connective tissue and osteoblast between implant, instead it grows inside the implant (fig. 6A,E,I). Even though, the control group has better proliferation quality of connective tissue and osteoblast (fig. 6B,F,J,D,H,L). On the other hand, in the HA-C group, the proliferation of connective tissue was found only on 30 days after implantation (fig. 6C). Previous study was also described that the HA-TCP implants in rabbit bone have same microscopic morphology characteristics with our present study. [24]

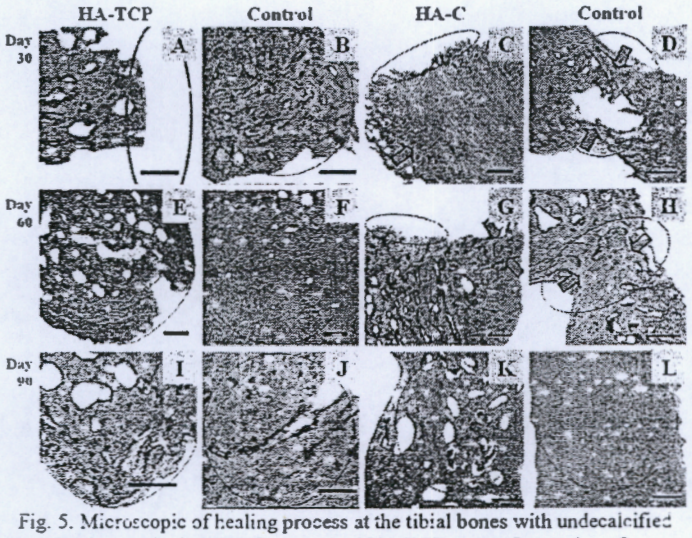

Fig. 5. Microscopic of healing process at the tibial bones with undecalcified evaluation method. A,E,I. Represented HA-TCP group. Increasing of new bone formation with trabecular form (→) after implantation. C,G,K. Represented HA-C group. There were no new bone formation or trabecular form (→) until day 90 implantation. B,F,J and D,H,L. Control each implant group. Defect area (○) of implant or hole at the control group. Bar = 0.5 mm

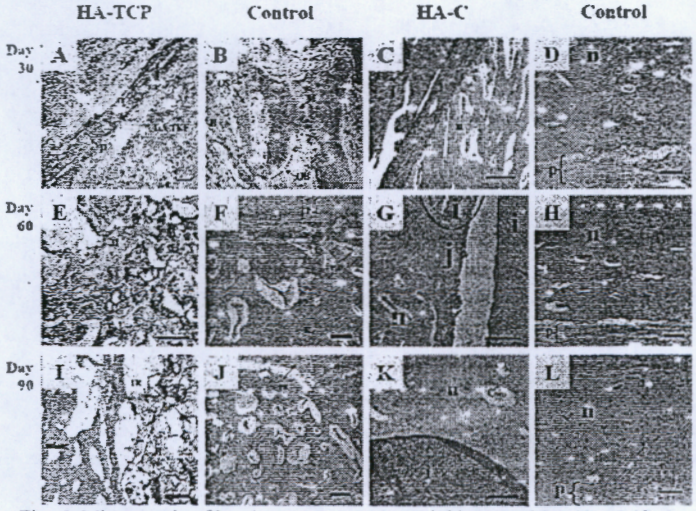

Fig. 6. Microscopic of healing process at the tibial bones with undecalcified evaluation method. A,E,I. Represented HA-TCP group. C,G,K. Represented HA-C group. B, F,J and D,H,L. Represented control each implant group. HE colored. Bar = 10μm. i = implant, j = connective tissue, n = *woven bone*, p = periosteum, s = bone marrow region. c = cartilage, o = osteogenic cells, and v = vascularisation.

Bone Healing and Bone Remodeling

Drilled bone healing has the same process of bone fracture. The process of bone graft incorporation in a spinal fusion model is similar to the bone healing process that occurs in fractured long bones.[25] Fracture healing restores the tissue to its original physical and mechanical properties and is influenced by a variety of systemic and local factors. Healing occurs in three distinct but overlapping stages: 1) the early inflammatory stage; 2) the repair stage; and 3) the late remodeling stage.[26,27]

In the inflammatory stage, a hematoma develops within the fracture site during the first few hours and days. Inflammatory cells (macrophages, monocytes, lymphocytes, and polymorphonuclear cells) and fibroblasts infiltrate the bone under prostaglandin mediation. This results in the formation of granulation tissue, ingrowth of vascular tissue, and migration of mesenchymal cells. The primary nutrient and oxygen supply of this early process is provided by the exposed cancellous bone and muscle. The use of antiinflammatory or cytotoxic medication during this 1st week may alter the inflammatory response and inhibit bone healing.[21,22]

As vascular ingrowth progresses, a collagen matrix is laid down while osteoid is secreted and subsequently mineralized, which leads to the formation of a soft callus around the repair site. In terms of resistance to movement, this callus is very weak in the first 4 to six weeks of the healing process and requires adequate protection in the form of bracing or internal fixation. Eventually, the callus ossifies, forming a bridge of woven bone between the fracture fragments. Alternatively, if proper immobilization is not used, ossification of the callus may not occur, and an unstable fibrous union may develop instead.[22]

IV. Conclusion

The US image has the ability detect the early bone remodeling i.e. inflammation process on the implantation site. On the other hand, the CR can describe gross imaging bones implant and the bone remodeling for a long term period. B-mode US, CR and the morphological evaluations showed that the healing process on control bone was faster than those of both types implanted bone graft i.e HA-TCP and HA-C. Even though HA-TCP has better biocompatibility, biodegradability, bioresorbability, bioactivity and osteo conductivity properties compared with HA-C.

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