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Research Article

The Role of Cuttlebone and Cuttlebone Derived Hydroxyapatite with Platelet Rich Plasma on Tibial Bone Defect Healing in Rabbit: An Experimental Study

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Abstract

Today marine-derived biologic scaffolds are popular due to their biocompatibility and high regenerative potential. Previous studies prepared hydroxyapatite from cuttlebone, the internal shell of cuttlefish; However, its biocompatibility and bioactivity has not been fully studied especially *in-vivo*. The aim of this study was to evaluate cuttlebone-derived hydroxyapatite in-vivo potential and possible synergistic effect of platelet rich plasma with this scaffold in promoting bone healing. Hydroxyapatite was prepared from cuttlebone *(Sepia officinalis)* via hydrothermal transformation. The conversion and microstructure of prepared material was assessed by scanning electron microscopy (SEM) and x-ray diffraction (XRD) analysis. Fifteen male white New Zealand rabbits were randomly divided to 6 groups each containing 5 limbs. In order to reduce sample size right and left pelvic limbs of rabbits were used as separate groups. Full thickness bi-cortical defects were created bilaterally in proximal tibia. The defect was left untreated in negative control group. In experimental group I to V the defect was filled with platelet rich plasma, raw cuttlebone, raw cuttlebone combined with platelet rich plasma, cuttlebone derived hydroxyapatite and cuttlebone derived hydroxyapatite combined with platelet rich plasma, respectively. Histopathological evaluation was performed on specimens received on day 56. Bone healing was assessed according to union, spongiosa, cortex and bone marrow indices. Our results demonstrated that Group I was superior to negative control group in defined indices. Groups IV and V showed preferable outcomes regarding to union and cortex indices in comparison to groups II and III. Also acceptable degree of spongiosa formation was observed in all groups. Cuttlebone derived hydroxyapatite could be an appropriate biomaterial to stimulate bone formation and enhance bone regeneration. Furthermore platelet rich plasma was successful in advancement of bone marrow formation.

Keywords: *Bone defect, Cuttlebone, Hydroxyapatite, Platelet rich plasma, Rabbit*

Tavşanlarda Mürekkepbalığı Kabuğu ve Kan Pulcuğundan Zengin Plazma İle Birlikte Mürekkep Balığı Kaynaklı Hidroksiapatitin Tibial Kemik Hasarının İyileştirilmesindeki Rolü: Deneysel Bir Çalışma

Özet

Günümüzde deniz ürünleri kaynaklı doku yapı iskeleleri, biyouyumlu ve yüksek yenilenme potansiyeline sahip olmaları nedeniyle popülerdirler. Mürekkep balığının iç kabuğu olan Mürekkepbalığı kabuğundan elde edilmiş hidroksiapatit ile yapılmış önceki çalışmalarda kabuğun biyouyumluluğu ve biyoaktivitesi özellikle in vivo olmak üzere tam olarak çalışılmamıştır. Bu çalışmanın amacı, mürekkep balığı kaynaklı hidroksiapatitin *in vivo* potansiyelini ve bu doku iskelesi ile kan pulcuğundan zengin plazmanın kemik iyileşmesini etkilemedeki sinerjik etkisini araştırmaktır. Hidroksiapatit mürekkepbalığı *(Sepia officinalis)* kabuğundan hidrotermal transformasyon ile elde edildi. Hazırlanan materyalin dönüştürme ve mikroyapısı Tarayıcı elektron mikroskop (SEM) ve x ışını kırımı (XRD) analizi ile kontrol edildi. On beş erkek beyaz Yeni Zelanda tavşanı rastgele olarak her birinde 5 bacak olacak şekilde toplam 6 gruba ayrıldı. Hayvan sayısını azaltmak adına sağ ve sol arka bacakları ayrı gruplar olarak kullanıldı. Proksimal tibiada bilateral olarak tüm katman biyokortikal hasar oluşturuldu. Negatif kontrol grubunda hasar tedavi edilmeksizin bırakıldı. Oluşturulan hasar I'den V'e kadar olan deneysel gruplarda sırasıyla kan pulcuğundan zengin plazma, işlenmemiş mürekkepbalığı kabuğu, işlenmemiş mürekkepbalığı kabuğu ile birlikte kan pulcuğundan zengin plazma, mürekkepbalığı kaynaklı hidroksiapatit ve mürekkepbalığı kaynaklı hidroksiapatit ile birlikte kan pulcuğundan zengin plazma ile dolduruldu. 56. günde alınan örneklerde histopatolojik inceleme gerçekleştirildi. Kemik iyileşmesi birleşme, spongioz, korteks ve kemik iliği belirteçleri göz önüne alınarak değerlendirildi. Elde edilen sonuçlar, göz önüne alınan belirteçler yönünden Grup I'in negatif kontrole göre daha üstün olduğunu gösterdi. Grup IV ve V'de grup II ve III ile karşılaştırıldığında birleşme ve korteks belirteçleri bakımından tercih edilebilir sonuçlar oluştuğu gözlemlendi. Tüm gruplarda kabul edilebilir düzeyde spongioz oluşumun gerçekleştiği gözlemlendi. Mürekkepbalığı kaynaklı hidroksiapatit kemik oluşumunu uyarmak ve kemik rejenerasyonunu geliştirmede uygun bir biyomateryal olabilir. Ayrıca kan pulcuğundan zengin plazmanın kemik iliği oluşumunu geliştirmede başarılı olduğu gözlemlendi.

Anahtar sözcükler: *Kemik hasarı, Mürekkepbalığı kabuğu, Hidroksiapatit, Kan pulcuğundan zengin plazma, Tavşan*

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INTRODUCTION

Bone is a dynamic tissue rich in blood vessels that acts as a structural and functional support in vertebrate's body. This specialized hard connective tissue undergoes remodeling and chemical exchange with other parts of body constantly [1-5]. The majority of bone injuries go through a gradual healing process without any scar formation and with considerable resumption of bone characteristics so that eventually the newly formed bony tissue is indistinguishable from peripheral healthy bone; nevertheless in extensive bone injuries like traumas, tumors and skeletal abnormalities healing is not successful because the extent of damage exceed the regenerative potential of bone. From past to now researchers have been looking for appropriate methods to potentiate and accelerate healing process or to replace the lost bone [3-8]. In different clinical conditions there are several therapeutic options available in order to reconstruct the defected bone; each of which has advantages and disadvantages [8,9]. Among these, bone grafts have been popular to researchers. Although autologous bone grafts have been mentioned as a gold standard, disadvantages of this method such as prolonged surgery time, pain and hemorrhage has limited its application lately [6,8,9]. Ideal bone substitutes should be non-immunogenic, bioactive, osteoconductive and osteoinductive, biodegradable, sterilizable, thermally non-conductive, traceable in vivo, readily available and economical [7-9]. Hydroxyapatite the main mineral component of hard tissues and one of the most stable forms of calcium phosphate has been utilized widely as a bone substitute in orthopedic and maxillofacial surgery in three past decades. It is a porous material that makes vascular ingrowth possible and provides oxygen and nutrients for cells [8-13]. Nowadays researchers are looking for natural materials considering them superior and more desirable [6,8,14]. Heretofore several raw materials such as eggshell and animal bone have been used to prepare hydroxyapatite; however, religious and social limitations along with disease transmission have made the use of these sources restricted [10,12,13,15]. Marine sources are novel in this field. Aquatic living organisms such as Coral, Nacre and Cuttlefish have been used in order to enhance bone regeneration recently [16,17]. Cuttlebone (CB) is the hard internal structure of cuttlefish consists of crystals of calcium carbonate (Aragonite) which plays essential role in protecting organism vital organs and act as a floating tank besides. It is biocompatible, osteoconductive and has plasticity in regard to morphology and mineral composition [10,13,18-20]. In addition, this structure is available in many oceans and seas worldwide with low expense. Parallel interconnected sheets give CB porosity. The pores diameter is in the range of 200-600 micrometer and therefore they are optimum for new bone formation and neovascularization [19,21]. The special structure and unique characteristics have encouraged researchers to try raw CB or cuttlebone derived hydroxyapatite (CBHA) in the field of tissue engineering and bone healing. Different methods have been used to prepare hydroxyapatite from CB recently which the most popular is hydrothermal synthesis. Time and temperature of the reaction are the main variables in this process [10,13,14,20,22,23]. Another biologic source that has widely introduced lately is platelet rich plasma (PRP); defined as a fraction of blood which platelet concentration is above baseline in it. PRP have been applied as an economical angiogenic orthobiologic in association with ceramics such as hydroxyapatite and majority of studies reported promising results; however some found opposite outcome so its regenerative capability is still controversial [24-27]. The aim of present study was to evaluate *in-vivo* effectiveness of raw CB and CBHA prepared by hydrothermal synthesis along with PRP in bone repair and to investigate probable synergism between these biomaterials.

MATERIAL and METHODS

Hydroxyapatite Preparation

CB was extracted from Cuttlefish (Sepia pharaonis from Persian Gulf), washed with distilled water and dried. The dorsal shield was removed and the lamellar part was cut into small blocks of about 1* 1*1 cm^2 by lancet and to remove organic residues immersed into 5% NaClO for 48 h. For hydrothermal synthesis the previously described method was slightly modified [22]. Briefly the required volume of 0.6 M aqueous solution of $NH_4H_2PO_4$ was added to yield a molar ratio of Ca/P = 1.67. The mixture was then sealed in a Teflon lined stainless steel pressure vessel and heated at 200°C in an electric furnace for 24 h. The resultant was dried under airflow prior to use. The raw CB was sterilized with Υ irradiation prior to *In vivo* implantation *(Fig. 1A-D)*.

Characterization of CB and CBHA

The microstructure and surface morphology of CB and CBHA were examined via SEM (SERON AIS-2100, Amirkabir University of technology, Tehran, Iran) after sputter coating with gold. To evaluate the composition of the raw and converted material, XRD analysis was performed. XRD patterns collected from 10-90° 2Θ at a 0.04°/min scanning rate.

PRP Preparation

After clipping and scrubbing the thoracic region adjacent to heart, the animals were anesthetized and positioned laterally and 8 mL fresh blood was obtained by intracardiac application of a long needle and transferred into Acid Citrate Dextrose containing sterile vacutainer immediately. The initial platelet count of blood samples were analyzed by veterinary automatic cell counter (NIHON KOHDEN, Japan) and the samples were centrifuged at 2400 rpm (SIGMA, Germany) for 20 min primarily in order to separate plasma. Separated plasma then centrifuged at 3600 rpm for 15 min once more. After this stage the supernatant plasma was

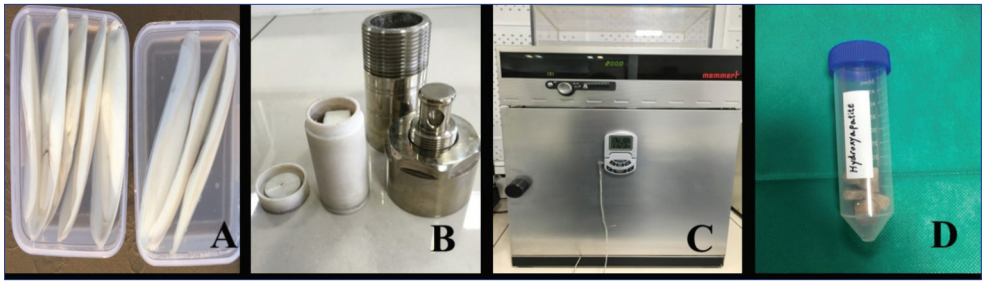


Fig 1. Hydroxyapatite preparation (A-D) A: cuttlebone extracted from cuttlefish, B: Teflon lined stainless steel pressure vessel, C: Electric furnace, D: Prepared hydroxyapatite

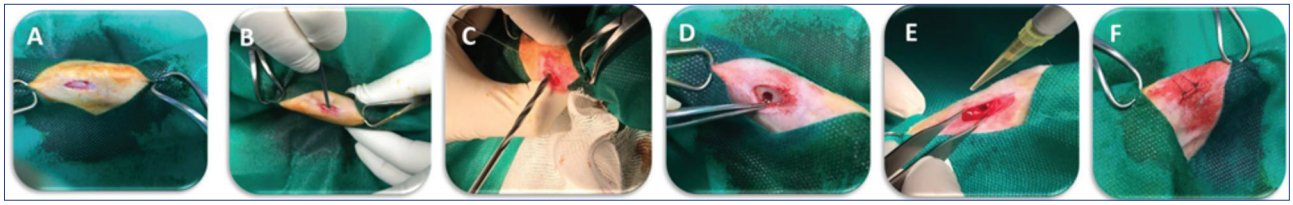


Fig 2. Surgical procedure A: Skin incision. B: Periosteal elevation and bone exposure. C and D: Tibial drill hole in order to make a defect. E: Platelet rich plasma application. F: Subcutaneous tissue and skin suture

removed and the remaining volume (about 0.5 mL) was collected and accounted as PRP. Finally the platelet counts were measured again and if the count was 3-5 times higher than the baseline count of the platelets, the samples were considered acceptable for the experiment.

In Vivo Testing, Fluoroscopy and Histopathological Examination

The animal research protocol was reviewed and approved by Iran society for the prevention of cruelty to animals (SPCA) and met Iranian Laboratory animals Ethic frameworks according to reference number IAEC 4-08/2. Fifteen adult male New Zealand white rabbits with similar average weight and age were kept under same environmental and nutritional conditions for two weeks for adaptation. Animals were randomly divided into 6 groups, including negative control group (untreated), group I (treated with PRP), group II (treated with raw CB), group III (treated with raw CB and PRP), group IV (treated with CBHA) and group IV (treated with CBHA and PRP). In order to involve fewer animals in the study we designed to use both legs of each animal; therefore, the animals left and right legs were clipped and scrubbed from the mid-shaft of the femur to tibia. Anesthesia was inducted in all groups with a combination of 40 mg/kg ketamine hydrochloride (10%®, Alfasan, Woerden, Netherlands) and 0.2 mg/kg medetomidine hydrochloride (Dorben Vet®, Spain SYVA s.a.u, Espain) and maintained with 3% Isoflurane. The animals were positioned in dorsal recumbency and the skin was incised in the proximal and medial region of the crus for about 2 cm *(Fig. 2A)*. After subcutaneous dissection, bone was exposed, the periosteum was pulled over *(Fig. 2B)* and a defect was created in tibia by 3.5 millimeter diameter slow speed orthopedic drill concomitant with irrigation in such a way that drill was entered from internal surface of the bone and moved out from the external surface *(Fig. 2C,D)*. In negative control group (left legs of 5 rabbits), the defect was left untreated, the periosteum was returned to its place and subcutaneous tissue and skin was sutured with continuous and interrupted suture patterns with 4-0 polyglactin 910 and 4-0 nylon, respectively *(Fig. 2F)*. In group I (right legs of same rabbits in negative control group), PRP was applied in the defect *(Fig. 2E)* after similar preparation and surgical procedure. CB was used as defect filler in group II (left legs of another 5 rabbits) and CB and PRP in group III (right legs of same rabbits). In group IV (left legs of another 5 rabbits) and v (right legs of same rabbits), CBHA and CBHA and PRP was implanted into the tibial bone defect respectively. Post-operative cares were included broad spectrum antibiotic and analgesic medications (5 mg/kg Enrofloxacine and 0.3 mg/kg meloxicam, Razak laboratory, Tehran, Iran) and adequate food and water intake. It should be noted that no considerable pain and discomfort was observed in any animal after surgery, because all animals were able to eat and drink adequately and bear weight. After 14 days the skin sutures were removed. Fluoroscopy imaging was done on day zero, immediately after surgery *(Fig. 3A,B)* to check bone integrity and on day 56 after surgery *(Fig. 3C,D)* in order to ensure the bone is unbroken and the implants are in place properly. The animals were euthanized 8 weeks after implantation and the defect sites along with the surrounding bone areas were dissected from the host bone. Specimens were put in 10% formalin buffer for fixation and a combination of 8% formic acid-8% hydrochloric acid for decalcification. After decalcification the specimens were dehydrated in a serial increasing concentration of ethanol, embedded in paraffin and sectioned. The sections were stained using hematoxylin and eosin and observed via light microscopy. Four indices (Union, Spongiosa, Cortex and Bone marrow) were defined for evaluation of bone healing according to Korkmaz et al.[28] study.

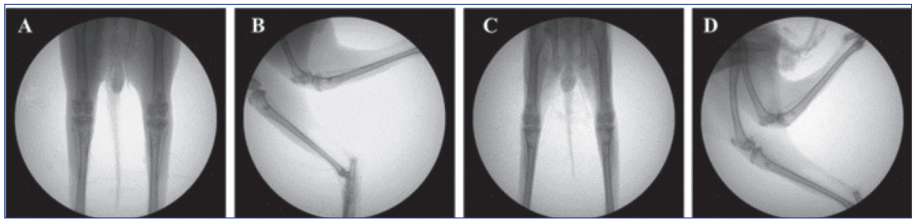


Fig 3. A and B: Fluoroscopic views of limbs on day zero. C and D: Fluoroscopic views of hind limbs on day 56

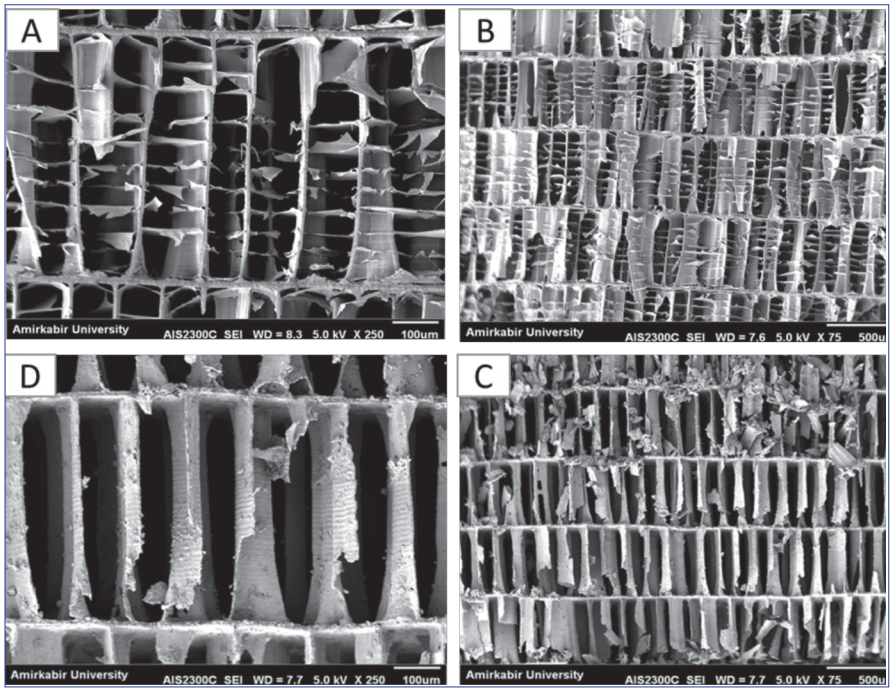


Fig 4. SEM image showing microstructure and surface characterization of raw cuttlebone (A, B) and hydrothermally converted hydroxyapatite (C, D); SEM, scanning electron microscopy

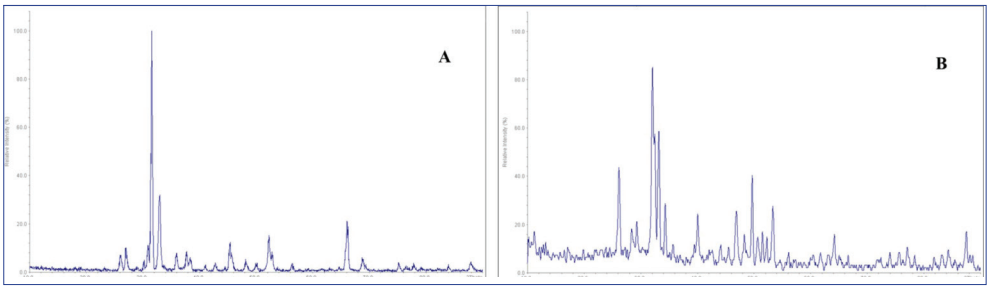


Fig 5. XRD pattern of raw cuttlebone (A) and hydrothermally converted hydroxyapatite (B).
XRD, x-ray diffraction

RESULTS

Characterization of CB and CBHA

The SEM image from raw CB demonstrated porous structure (pore size approximately 100 µm) with several interconnected chambers separated by vertical pillars. SEM micrograph of CBHA revealed similar structure; this indicates that most of the characteristic surface morphologies were preserved through hydrothermal synthesis process. *Fig. 4A,B* shows SEM micrographs of raw CB and *Fig. 4C,D* shows SEM micrographs of prepared CB. XRD patterns of CB before (A) and after (B) the conversion are shown in *Fig. 5*. XRD pattern of raw CB fairly resembled aragonite (crystal form of calcium carbonate). Conversion to hydroxyapatite was confirmed by XRD analysis. Close agreement was present between peeks of the specimen and the standard hydroxyapatite.

Histopathological Study

Each group received a score for defined indices *(Table 1)*. Group I (defect filled with PRP) was superior to negative control group in all of the investigated indices. Hydroxyapatite groups (IV and V) showed preferable outcomes regarding to union and cortex indices. Reorganized spongiosa formation and complete reorganized formation was identified in respect of spongiosa in all groups except negative control. PRP stimulated bone marrow formation according to results. In negative control group, the defect site was covered with abundant amount of fibrous connective tissue and some amount of cartilaginous callus

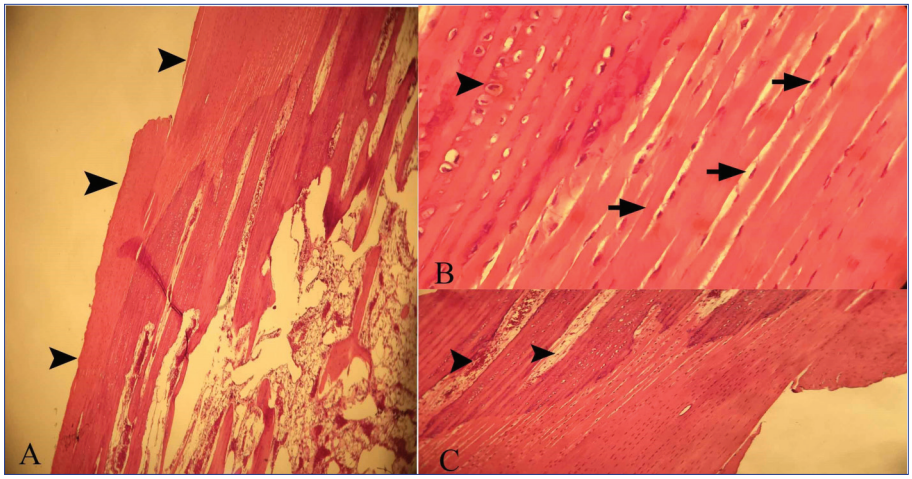


Fig 6. H&E stained images of defect site in negative control group on day 56. A: Considerable amount of fibrous connective tissue *(arrowhead)* and some amount of cartilaginous callus are visible in the defect site (×4). B: Fibrous connective tissue containing collagen fibers and fibrocyte nucleus *(arrow)* and cartilaginous callus containing chondrocytes *(arrowhead)* is evident (×40). C: Bone marrow cavities *(arrowhead)* (×10)

Table 1. *Histopathological results*

Index	Evaluation Scale	Score	Experimental Groups					
			Negative Control	Group I	Group II	Group III	Group IV	Group v
Union	No sign of union	0	1	2	3	3	4	4
	Fibrous union	1						
	Osteochondral union	2						
	Bone union	3						
	Complete reorganization	4						
Spongiosa	No sign of cellular activity	0	2	3	3	4	4	4
	Early bone formation	1						
	Active new bone formation	2						
	Reorganized spongiosa formation	3						
	Complete reorganized formation	4						
Cortex	Absence of cortex	0	2	3	3	3	4	4
	Early detection	1						
	Initiation of formation	2						
	Reorganization in majority	3						
	Complete organization	4						
Bone marrow	Not available	0	2	3	4	4	3	4
	Detection of fibrinous material	1						
	Defect occupying more than half	2						
	Fully occupying the red Bone marrow	3						
	Adult type fatty marrow	4						
Summation		16	7	11	13	14	15	16

was present. In addition collagen containing connective tissue and chondrocyte containing callus were present along with scant cancellous tissue *(Fig. 6)*. In group I, the defect site was enveloped with osteocartilaginous callus and chondrocytes and osteocytes were present. Bone marrow cavities were filled with hematopoietic bone marrow *(Fig. 7)*. In groups II and III, a thin osseous tissue had coated the defect site and inflammatory reaction was seen in the region of bone marrow. Also inflammatory response characterized by mononuclear phagocytic cells in the periphery of CB remnants was detected *(Fig. 8, Fig. 9)*. In group IV, new bone formation was determined at the defect site. Furthermore blood was noticed in bone marrow cavities concomitant with hydroxyapatite residues *(Fig. 10)*. In the last group (V), formation of Haversian systems was seen and hydroxyapatite residues were observed in bone marrow cavities *(Fig. 11)*.

Fluoroscopy

Proximal tibia was broken in one rabbit in group II on

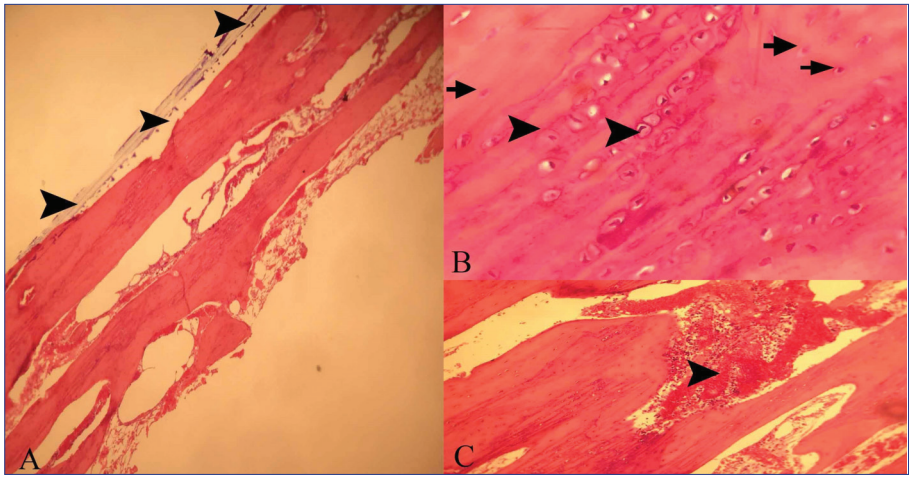
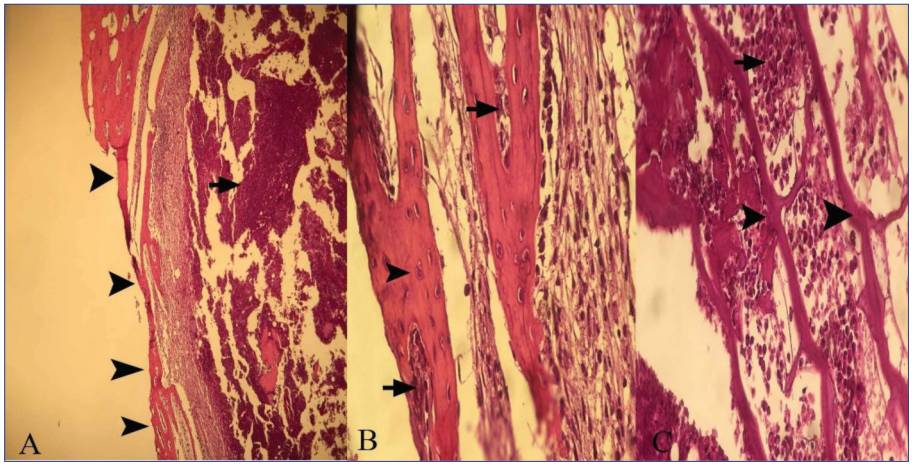


Fig 7. H&E stained images of defect site in group I on day 56. A: The defect site is covered by a large quantity of osteo-cartilaginous callus *(arrowhead)* (×4). B: Chondrocytes *(arrowhead)* and Osteocytes *(arrow)* are visible at the healing site (×40). C: Hematopoietic bone marrow *(arrowhead)* has filled bone marrow cavities (×10)

Fig 8. H&E stained images of the defect site in group II on day 56. A: Arrowheads indicate a thin covering of osseous tissue and the arrow indicates inflammatory reaction in the bone marrow region (×4). B: Endosteal osteoblasts and lacunar hypertrophic osteocytes are evident (×40). C: Cuttlebone remnants *(arrowhead)* surrounded by mononuclear inflammatory response (×40)



fluoroscopy imaging done on day zero, after surgery *(Fig. 12,A)*. Fixation by intramedullary pin was done *(Fig. 12,B)*. Fifty six days after surgery, union was achieved *(Fig. 12,C)* and the pin was removed after euthanasia. In the other groups the bone integrity was preserved, no fractures were seen and all implants were in their place *(Fig. 3)*.

DISCUSSION

Nowadays bone substitutes have opened new insights in bone regeneration by reducing the limitations of bone graft such as low availability, high cost, zoonotic diseases transmission, immune reactions and so on [4,7,8]. In general natural and synthetic scaffolds are available; each has specific advantages and disadvantages [6,8]. Biocompatibility, biodegradation and regenerative characteristics including osteoinduction, osteoconduction and osteogenesis are the most important advantages of natural scaffolds over synthetic ones [8]. Hydroxyapatite is well-known because of high similarity to mineral bone composition [8-10,29]. Some natural sources have capacity to be converted into hydroxyapatite, for example Ni et al.[30] obtained hydroxyapatite from nacre, and Gao et al.[31] obtained it from coral. In addition different species of wood were pyrolized and its carbonate residues saturated with calcium salts to achieve bioactive hydroxyapatite [9]. CB is a unique marine biomaterial potentially convertable to hydroxyapatite that has attracted attention of researchers currently [10,13,14,19,20,22]. CB compatibility with bone mineral composition was demonstrated by quantitative analysis of sodium, magnesium, pottasium and calcium ions in CB and human elbow bone [18,19]. Many researches focused on evaluation of CB effect on bone regeneration, alone or in combination with grafts, growth factors and cell culture [10,18,19,29,32,33]. For instance CB mechanical and biological characteristics as an bioactive acrylic bone cement was assessed previously *in vivo* and efficient implant integration with bone was reported and there was no evidence of secondary infection [18]. In another study, Kim [20] and colleagues investigated attachment and differentiation of mesenchymal cells on CB dorsal shield and lamellar part and concluded that lamellar matrix permits better cellular penetration than dorsal shield, although its a brittle structure and needs more investigations in order to increase strength. Yi [32] and colleagues showed synergistic effect of CB, autologous bone marrow and sodium hyaloronate in healing of radial bone defect in rabbit. Liu et al.[33] confirmed the positive effect of BMP impregnated CB on osteogenesis and neovascularization of rat calvarial defect by histopathological examination.

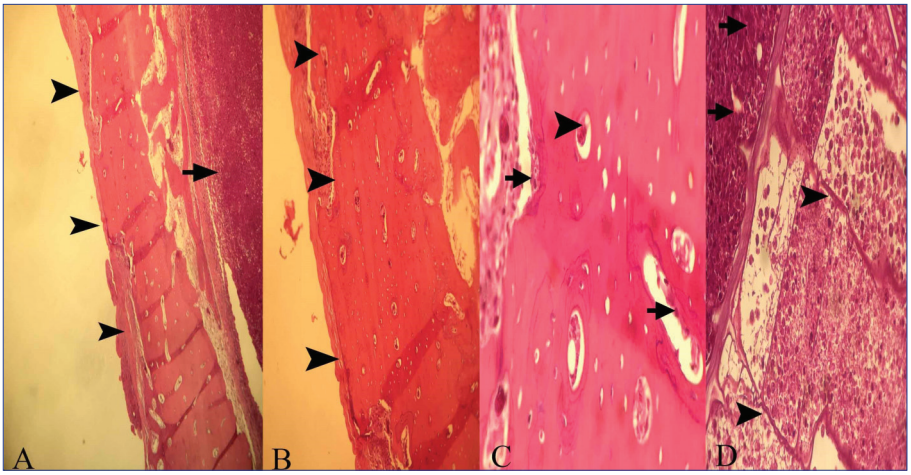
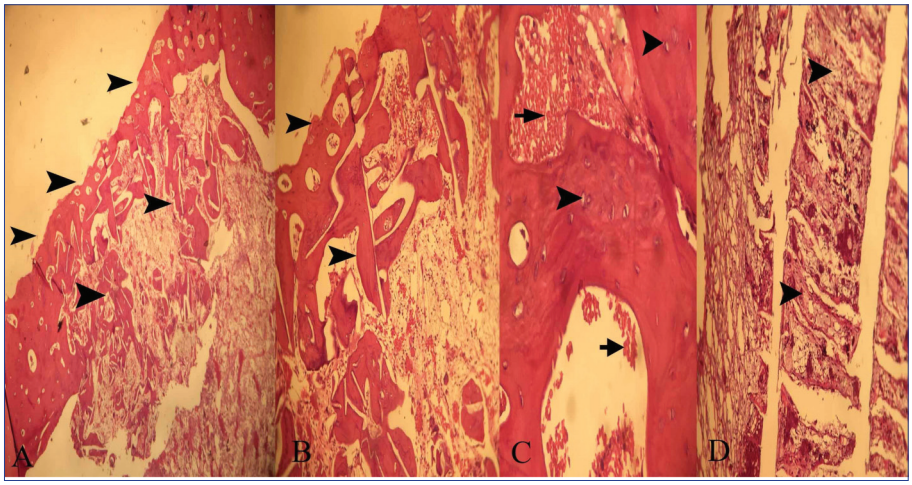


Fig 9. H&E stained images of defect site in group III on day 56. A: Marrow derived inflammatory reaction *(arrow)* and osseous tissue *(arrowhead)* are observable at the defect site (×4). B: *Arrowheads* indicate osseous callus at the healing site (×40). C: Osteoblasts *(arrow)* are noticeable in the endosteum and bone marrow cavities (×40). D: Cuttlebone remnants are encompassed with mononuclear inflammatory response *(arrow)* (×40)

Fig 10. H&E stained images of defect site in group IV on day 56. A: Arrowhead demonstrates osseous tissue at defect site (×40). B: New bone formation *(arrowhead)* is indicated (×40). C: Blood-filled bone marrow cavities *(arrow)* and osteocytes *(arrowhead)* are visible (×40). D: Hydroxyapatite residues are present in bone marrow cavities (×40)



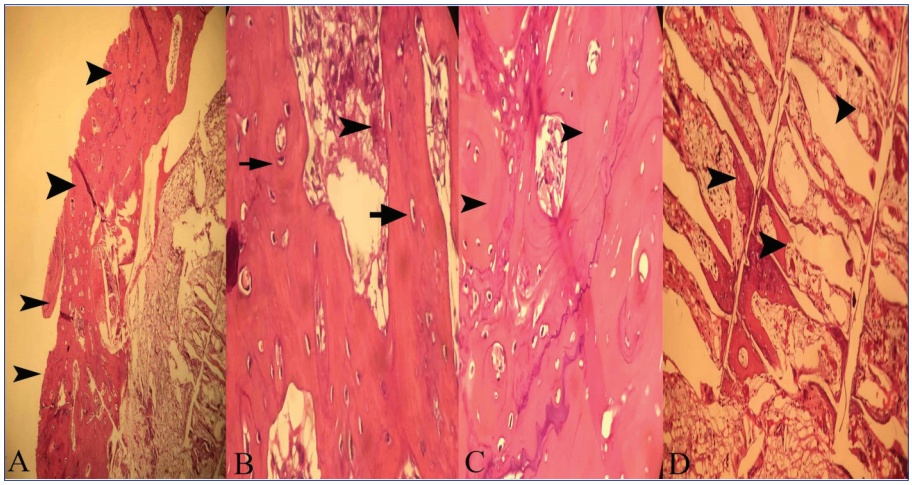


Fig 11. H&E stained images of defect site in group V on day 56. A: Osseous tissue *(arrowhead)* had covered the defect site (×4). B: *Arrows* are indicative of osteoblasts in the endosteum and *arrowhead* is indicative of hypertrophic osteocytes in the lacuna (×40). C: Haversian systems *(arrowhead)* are observable in newly formed osseous tissue (×40). D: Hydroxyapatite remnants *(arrowhead)* are visible in bone marrow cavities (×40)

In similar clinical setting, Dogan and Okamus [19] compared raw CB, demineralized bone matrix, bovine cancellous bone and tricalcium phosphate in regeneration of metaphyseal radial bone defect in rabbit. CB was inferior to other groups radiologically but it presented the best result in histopathological evaluations. In our study fracture of proximal tibia was likely due to excessive pressure at the site of drilling and being close to bone cortex. However, fracture was observed only in one rabbit of experimental group II. According to fluoroscopic views on day 56 *(Fig. 12 C)*, fracture line of proximal tibia in mentioned rabbit was disappeared and healing was achieved. Since CB has many positive characteristics and is readily available in our country by low expence we used it as a biomaterial in this study. CB showed promissive effects on bone healing purely and also incombination with PRP, however it's effect

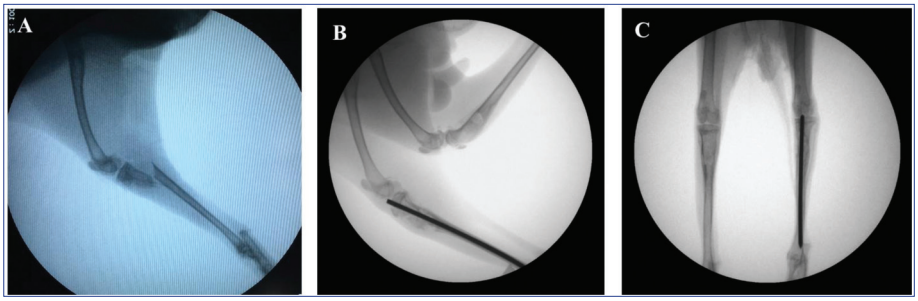


Fig 12. Fluoroscopic views of broken limb of the rabbit in group II. A: Immediately after surgery, B: Fixation of the fracture by intramedullary pinning, C: Union on day 56

was inferior in comparison to CBHA based on our histopathological results *(Table 1)*. Other studies utilized CB after processing it into hydroxyapatite [10,13,14,22,23]. Rocha et al.[21] produced hydroxyapatite from CB by hydrothermal synthesis for the first time in literature review. Ivankovic et al.[22] synthetized hydroxyapatite in the temperature range of 140-220ºC for various times from 20 min to 48 h in order to provide a suitable scaffold for tissue engineering. They achieved complete conversion of aragonite into hydroxyapatite in 200ºC and 24 h. In present study hydroxyapatite blocks were prepared via hydrothermal synthesis in 200ºC, within 24 h in electric furnace and conversion was confirmed by XRD analysis *(Fig. 5)*. Porosity and the pores size are responsible for providing optimum environment for tissue regeneration and nutrient diffusion between cells and the neighboring area. These characteristics depend on the consumed material and the fabrication method extensively [3,6,8]. The pores provide supportive structure for cell growth, neovascularization and development of bone tissue [10]. Too small pores will be obstructed by cells and this will preclude the afformentioned processes; while big pores will decrease mechanichal strength beside establishing better interaction with the surrounding tissue [8]. It was reported that 100-400 micrometer pore size is ideal although 200-350 µm was reported also elsewhere [8-10]. CB is a porous material with the pore size between 200-600 µm and therefore its suitable for new bone formation and neovascularization [10,11,14,21]. In our study the pore size was measured approximately 100 µm. Initial porous interconnected structure was preserved under hydrothermal procedure according to SEM images *(Fig. 4)*. Acceptable results were reported from *in vitro* application of CBHA [10,14,34]. Buttistella et al.[34] evaluated osteogenic markers expression and MC3T3-E1 cells proliferation on CBHA. In another study Kim et al.[10] designed a scaffold by adding polycaprolactone to CBHA. The scaffold induced efficient cell response and showed amazing potential for application in tissue engineering. According to Hongmin et al.[14], CBHA supported human mesenchymal cell growth and was appropriate for filling bone defects. Although CB bioactivity *in vitro* has been showed, CB biocompatibility *in vivo* has not been fully studied. Hongmin et al.[14] implanted mice dorsal subcutaneous pockets with CB and CBHA and reported ectopic bone formation just in hydroxyapatite group. In contrast in present study, although hydroxyapatite showed superior bioactivity and regenerative properties, the effectiveness of pure CB on healing process could'nt be ignored. Kim et al.[10] compared *In vivo* bioactivity of CBHA and synthetic hydroxyapatite in rabbit calvarial bone deffect and higher bone formation and accelerated healing process was present in CBHA group [11]. In this study we evaluated CBHA in load axis of tibia. Histopathological examination showed that CBHA could induce new bone formation as a bone substitute in rabbit tibial bone defect. PRP, an inexpensive source rich in growth factors has emerged as an adjuvant therapy that may accelerate regenerative process recently [24-27]. Numerous experimental studies have applied PRP in association with bone grafts and substitutes; only some were successful [24,27,35]. Therefore despite PRP pervasive application, its clinical efficacy is yet questionable. The combination of hydroxyapatite and PRP was significantly superior versus 0.9% sterile salt solution and PRP in reconstruction of periodontal membrane lesions [27]. It is reported that human PRP combined with persian gulf coral can promote healing of rabbit radial bone defect [8]. A combination of autologous bone and PRP for alveolar cleft reconstruction was evaluated and no significant statistical difference was found between the two groups [27]. Because the effect of CBHA and PRP combination has not been investigated heretofore and also due to controversial regenerative potential of PRP we decided to assess PRP in experimental setting once more. PRP was applied concurrently by the time of implantation of CB and CBHA and on our histopathological studies demonstrated that PRP is capable of promoting bone marrow formation and development during healing process. In present study the regenerative potential and biocompatibility of CB and CBHA was compared via introduction of these two material into rabbit tibial bone defect. We concluded that although raw CB itself is an admissible bone defect filler, the prepared hydroxyapatite is more promisive according to defined histopathologic indices. We introduced raw and processed CB as an accetable marine source that can induce new bone formation, but still further studies with farther number of animal models and longer evaluation period are required in order to assess this biomaterial precisely and follow the remodeling phase of the bone healing process.

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Conflict of Interest

None.

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