

Effects of combined hydroxyapatite and human platelet rich plasma on bone healing in rabbit model: radiological, macroscopical, histopathological and biomechanical evaluation

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Abstract Hydroxyapatite is an osteoconductive material used as a bone graft extender and exhibits excellent biocompatibility with soft tissues such as skin, muscle and gums, making it an ideal candidate for orthopedic and dental implants or components of implants. Synthetic hydroxyapatite has been widely used in repair of hard tissues, and common uses include bone repair, bone augmentation, as well as coating of implants or acting as fillers in bone or teeth. On the other hand, human platelet rich plasma (hPRP) has been used as a source of osteoinductive factor. A combination of hPRP and hydroxyapatite is expected to create a composite with both osteoconductive and osteoinductive properties. This study examined the effect of a combination of hydroxyapatite and hPRP

on osteogenesis in vivo, using rabbit model bone healing. A critical size defect of 10 mm long was created in the radial diaphysis of 36 rabbit and either supplied with hydroxyapatite-human PRP or hydroxyapatite or was left empty (control group). Radiographs of each forelimb were taken postoperatively on 1st day and then at the 2nd, 4th, 6th and 8th weeks post injury to evaluate bone formation, union and remodeling of the defect. The operated radiuses of half of the animals in each group were removed on 56th postoperative day and were grossly and histopathologically evaluated. In addition, biomechanical test was conducted on the operated and normal forearms of the other half of the animals of each group. This study demonstrated that hydroxyapatite-humanPRP, could promote bone regeneration in critical size defects with a high regenerative capacity. The results of the present study demonstrated that hydroxyapatite-hPRP could be an attractive alternative for reconstruction of the major diaphyseal defects of the long bones in animal models.

Keywords Hydroxyapatite · Human platelet-rich plasma · Radius · Bone healing · Rabbit

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Introduction

Evaluation of biomaterials for bone healing promotion is a key issue in orthopedic research surgery. Autogenous bone still remains the “gold standard” of bone

graft material in all facets of orthopedic surgery and is commonly used as a standard to which allografts and graft substitutes are compared (Alexander 1985; Alexander 1987; Brinker et al. 1997; Fitch et al. 1997; Fox 1984; McLaughlin and Roush 1998; Bigham et al. 2009). While application of the autografts in the bone defects is effective in diminishing the risk of the infectious disease transmission, they have also optimal osteoconductive, osteoinductive, and osteogenic properties. Moreover, there is no immune response after their implantation, and this criterion enhances their ability to incorporate into the new sites (Lohmann et al. 2001; Pokorny et al. 2003; Shafiei et al. 2009). However, a number of disadvantages such as morbidity in the donor site, the need for general anesthesia or sedation, and occasional requirement for more than one surgical field have also been described in utilization of the autogenous bone grafts. In addition, the graft survival is unpredictable, its resorption cannot be foretold and its availability is limited (Bauer and Muschler 2000; Keating and McQueen 2001; Dehghani et al. 2008). Therefore, several biocompatible materials have emerged as the substitutes of the autologous bone in the recent years. These can be classified into two major organic and synthetic groups. The biological biomaterials can be of allogeneic or homologous (human cortical bone and demineralised bone matrix or demineralized freeze-dried bone), heterologous, or xenogeneic (organic bovine, porcine, caprine, coral-derived hydroxyapatite) and replicating origin (morphogenetic proteins). The artificial or synthetic hydroxyapatite, bioglass and bioceramics are the most popular synthetic biomaterials used in Human and Veterinary Orthopedics (Esposito et al. 2006).

A number of growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor- β 1 (TGF- β 1), and insulin like growth factor (IGF) are available in PRP that have stimulating effect on healing of the bone defects. This stimulating effect is resulted due to chemotaxis induction as well as proliferation and differentiation of the osteoblasts and their precursors (Bostrom et al. 1999; Weibrich et al. 2002). An easy and more physiological way of application these growth factors to bone defects is via the use of platelet-rich plasma (PRP), a thrombocyte concentrate made up of autogenous blood (Marx et al. 1998; Weibrich et al. 2002).

Several investigations have previously demonstrated the positive effect of PRP on wound healing (McClain et al. 1996; Mustoe et al. 1991; Saba et al. 2002). A lot of studies have been conducted to investigate the effects of PRP upon regeneration of the bone defects (Marx et al. 1998; Aghaloo et al. 2002; Anitua 1999; Kassolis et al. 2000; Nash et al. 1994; Robiony et al. 2002; Rodriguez et al. 2003; Schlegel et al. 2004). However, the results of these studies are controversial. Marx et al. (1998) and Anitua (1999) used PRP for reconstruction of the maxillofacial defects in humans and found that PRP resulted in a quicker maturation of autogenous bone transplants and resulted to a higher bone density (Marx et al. 1998).

Further clinical investigations suggested an osteogenic potential of PRP but those experiments either did not include control groups (Kassolis et al. 2000; Robiony et al. 2002; Rodriguez et al. 2003) or could not identify any positive effect (Froum et al. 2002). It should be stated that not only the clinical data are contradictory, but in vivo experimental findings are also inconsistent. In a bone defect in the iliac crest of dogs, PRP combined with demineralized bone powder enhanced bone formation around the titanium implants (Kim et al. 2002). In a rabbit skull model, however, PRP did not influence bone healing (Aghaloo et al. 2002). In a similar study in pigs, PRP enhanced bone density temporarily when applied together with autograft but it was not effective when used in conjunction with a collagen scaffold containing additional osteoinductive proteins (Schlegel et al. 2004). Therefore, there are numerous biomaterials available for use to promote bone healing (Esposito et al. 2006), but the exact indication of each of them remains controversial.

No assessment has been made as yet of a combination of human plasma rich platelet (hPRP) with hydroxyapatite on the healing of the long bone defects. Our hypothesis is that the hPRP with other biomaterials such as synthetic hydroxyapatite can promote bone regeneration in radial bone defects in rabbit model. Therefore, hydroxyapatite was selected as a scaffold because of its interconnected porous architecture, high compressive breaking stress, proper biocompatibility and resorbability. The experiment was designed to compare the healing potential of hPRP delivered on a porous hydroxyapatite with that of the hydroxyapatite alone, while in the animals of the third group the defects were left empty.

Materials and methods

Animals and operative procedures

Thirty six New Zealand white rabbits, 12 months old, of both sexes, weighing 2.00 ± 0.50 kg, were kept in separate cages, fed a standard diet and allowed to move freely during the study. The animals were randomly divided into 3 equal groups as hydroxyapatite group ($n = 12$), hydroxyapatite-hPRP group ($n = 12$) and empty group ($n = 12$, control group). All the animals were anesthetized by intramuscular administration of 40 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. In all animals the right forelimb was prepared aseptically for operation. A 5 cm incision was made craniomedially over the skin of the fore limb and the radius was exposed by dissecting the surrounding muscles. A 10 mm segmental defect was then created in the mid portion of each radius as a critical size bone defect. In the animals of the hydroxyapatite group and hydroxyapatite-hPRP group, the bone defect was filled with hydroxyapatite segments (OS Satura[®], Isotis Co, Netherland). Four days after operation 1 ml hPRP was injected percutaneously into the defect of bones in the animals of the hydroxyapatite-hPRP group while the defects of the rabbits of the control group were left empty. The animals were housed in compliance with our institution's guiding principles "in the care and use of animals". The local Ethics Committee for animal experiments approved the design of the experiment

Preparation of PRP

Human PRP was prepared and supplied by the Shiraz Blood Bank Center. About 500 ml blood from a healthy donor was collected in 70 ml of anticoagulants (citrate–phosphate–dextrose [CPD]) and cooled to about 22°C. Within 24 h of extraction, the blood was separated through centrifugation into erythrocytes, buffy coat (leukocytes and thrombocytes) and plasma. From the buffy coat the leukocytes were removed through filtration, and the isolated fraction of platelets was human PRP. To obtain information on the increase in platelet concentration and the final concentration of platelets in the PRP of the blood, both whole blood and prepared PRP were subjected to platelet counts. Platelet counts were performed using a hematology analyzer (Advia 120, Bayer B.V.,

Mijdrecht, Netherlands). Number of platelets in the whole blood and PRP was $239 \times 10^9/l$ and $2,422 \times 10^9/l$ respectively.

Post operative evaluations

Radiological evaluation

To evaluate bone formation, union and remodeling of the defect, radiographs of each forelimb was taken postoperatively on 1st day and then at the 2nd, 4th, 6th and 8th weeks post injury. The results were scored using the modified Lane and Sandhu scoring system (Lane and Sandhu 1987; Table 1).

Gross evaluation

The operated radial bones of rabbits were removed on 56th postoperative day; at this time the operated radius was evaluated for gross signs of healing. Examination and blinded scoring of the specimens included presence of bridging bone, indicating a complete union (+3 score), presence of cartilage, soft tissue or cracks within the defect indicating a possible unstable union

Table 1 Modified Lane and Sandhu radiological scoring system

Bone formation	
No evidence of bone formation	0
Bone formation occupying 25% of the defect	1
Bone formation occupying 50% of the defect	2
Bone formation occupying 75% of the defect	3
Bone formation occupying 100% of the defect	4
Union (proximal and distal evaluated separately)	
No union	0
Possible union	1
Radiographic union	2
Remodeling	
No evidence of remodeling	0
Remodeling of medullary canal	1
Full remodeling of cortex	2
Total point possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum score	10

(+1 or +2 score), or complete instability at the defect site indicating no union (0 score).

Histopathological evaluation

Eight weeks after operation the rabbits were euthanized for histopathological and biomechanical evaluation. The histopathological evaluation was carried out on six rabbits of each group randomly. The right forelimb of each animal was harvested and dissected free of soft tissues. Sagittal sections containing the defect were cut with a slow speed saw. Each slice was then fixed in 10% neutral buffered formalin. The formalin-fixed bone samples were decalcified in 15% buffered formic acid solution and processed for routine histological examination. Two 5 micron in thickness sections were cut from the centers of each specimen and were stained with hematoxylin and eosin. The sections were blindly evaluated and scored by two pathologists according to the Emery's scoring system (Emery et al. 1994) and based on this scoring system the defects were evaluated as follows: When the gap was empty (score = 0), if the gap was filled with fibrous connective tissue only (score = 1), with more fibrous tissue than fibrocartilage (score = 2), more fibrocartilage than fibrous tissue (score = 3), fibrocartilage only (score = 4), more fibrocartilage than bone (score = 5), more bone than fibrocartilage (score = 6) and filled only with bone (score = 7).

Biomechanical evaluation

The biomechanical test was conducted on the injured and normal contralateral bones of each rabbit. The tests were performed using a universal tensile testing machine (Instron, London, UK; Oryan et al. 2008, 2011; Oryan and Shoushtari 2009). The three-point bending test was performed to determine the mechanical properties of bones. The bones were placed horizontally on two rounded supporting bars located at a distance of 30 mm, and were loaded at the midpoint of the diaphysis by lowering the third bar so that the defect was in the middle and had an equal distance from each grip. The bones were loaded at a rate of 10 mm/min until fracturing occurred. The behavior of each specimen under loading was characterized by determining the following parameters from the load deformation to destruction curve.

1. $\text{Tan-}\alpha$: the coefficient of inclination for the linear portion of the load-deformation curve, represents the index of stiffness of the material and is expressed as N/mm. It is easily calculated by measuring the slope of a line drawn as a tangent to the curve at any defined point. The slope gives the approximate stiffness of the preparation.
2. Ultimate strength: the highest registered load (N).
3. The specimen's extension at the ultimate strength region: the term "strain" means the fractional increase in length of the material due to an applied load. It is calculated by dividing the extension by the original length of the specimen. Strain is more useful than extension, because it minimizes the influence of length measurement error and does not depend on the specimen size.
4. Stress: Ultimate strength proportion to cross sectional area

The data derived from the load deformation curves were expressed as Mean \pm SEM for each group and maximum load, stiffness, stress and strain was measured and recorded.

Statistical analysis

The radiological, clinical and histopathological data were compared by Kruskal–Wallis, non-parametric ANOVA, when P values were found to be less than 0.05, then pair wise group comparisons was performed by Mann–Whitney U test. The biomechanical data were compared by a student's t test between the treated and normal limb data and one way ANOVA test was used for biomechanical analysis between the treated bones of all groups (SPSS version 17 for windows, SPSS Inc, Chicago, USA).

Results

Radiological findings

There was a significant difference in bone formation between the hydroxyapatite with hPRP-hydroxyapatite group on the 14th post injury days ($P < 0.05$). Bone healing in hPRP-hydroxyapatite group was superior to hydroxyapatite group. Superior bone formation was observed in hPRP-hydroxyapatite group in comparison with control group in 42nd post

injury days ($P < 0.05$). There was a significant difference in bone formation between the control and hydroxyapatite with hPRP-hydroxyapatite group on the 56th post injury days ($P < 0.05$). By day 56, there was 75–100% bone formation in the animals of the hydroxyapatite and hPRP-hydroxyapatite group compared to those of the control group that showed 50–75% bone formation (Table 2; Figs. 1, 2, 3).

Bone union had occurred in the hydroxyapatite and hPRP-hydroxyapatite rabbits by day 28th, 42nd and 56th post-injury, but not in controls group. In addition, bone union in the animals of the hydroxyapatite group by day 42nd and 56 post-injury was more prominent than the control ones. This trend continued with less union occurring in the animals of the control group (Tables 3, 4; Figs. 1, 2, 3).

Remodeling was superior in hPRP-hydroxyapatite group in comparison with the hydroxyapatite and control groups on 42th and 56th days post surgery. However, the animals of the Hydroxyapatite group showed better remodeling criteria on day 56 than those of the control group (Table 5; Figs. 1, 2, 3).

Gross and histopathological findings

The defect areas of the rabbits in all groups showed various amounts of new bone formation; however, the defects of the animals of the control group left blank or generally contained the least amounts of new bone and were often filled with a mixture of fibrous connective tissue and cartilage. The union

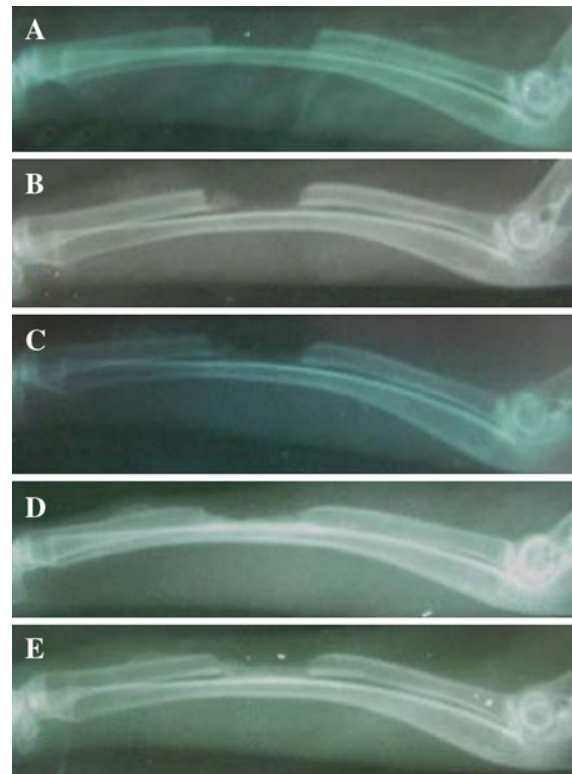


Fig. 1 Radiographs of treated forelimb in control group, on 1st day (a), 14th postoperative day (b), 28th postoperative day (c), 42nd postoperative day (d) and 56th postoperative day (e)

scores of the rabbits administered with hydroxyapatite or hydroxyapatite-hPRP were statistically superior to those of the animals of the control group

Table 2 Radiographical findings for bone formation at various post-operative intervals

Postoperative days	Med (min–max)			P^a
	Control (n = 12)	Hydroxyapatite (n = 12)	Hydroxyapatite-hPRP (n = 12)	
14	0 (0–1)	0 (0–0)	1 (0–1) ^b	0.02
28	1 (0–1)	1 (0–2)	1 (0–3)	0.2
42	1 (0–3)	2 (1–3)	3 (1–3) ^{c,d}	0.006
56	2 (1–3)	3 (2–4) ^e	3 (2–4) ^f	0.002

Significant P values are presented in bold face

^a Kruskal-Wallis non-parametric ANOVA

^b $P = 0.009$ (compared with hydroxyapatite by Mann–Whitney U test)

^c $P = 0.005$ (compared with control by Mann–Whitney U test)

^d $P = 0.03$ (compared with hydroxyapatite by Mann–Whitney U test)

^e $P = 0.005$ (compared with control by Mann–Whitney U test)

^f $P = 0.003$ (compared with control by Mann–Whitney U test)



Fig. 2 Radiographs of treated forelimb in hydroxyapatite group, on 1st day (a), 14th postoperative day (b), 28th postoperative day (c), 42nd postoperative day (d) and 56th postoperative day (e)

(Table 6). The union scores at macroscopic level correlated closely with the radiographic union score on day 56 post injury.

At histopathologic level, the defects of the animals of the hydroxyapatite and hydroxyapatite-hPRP group showed more advanced healing criteria than those of the control group (Table 6).

Fibrous nonunions or fibrocartilages in the defects of the animals of the control group were dominant and the lesions of these animals showed poor re-vascularization. Bridging callus or histological union did not develop in any of these defects. These criteria lead to very slow healing process in the animals of the control group (Fig. 4).

The defects of two rabbits of the hydroxyapatite group were filled with mature cortical bone and the lesion in the rest three from four rabbits was

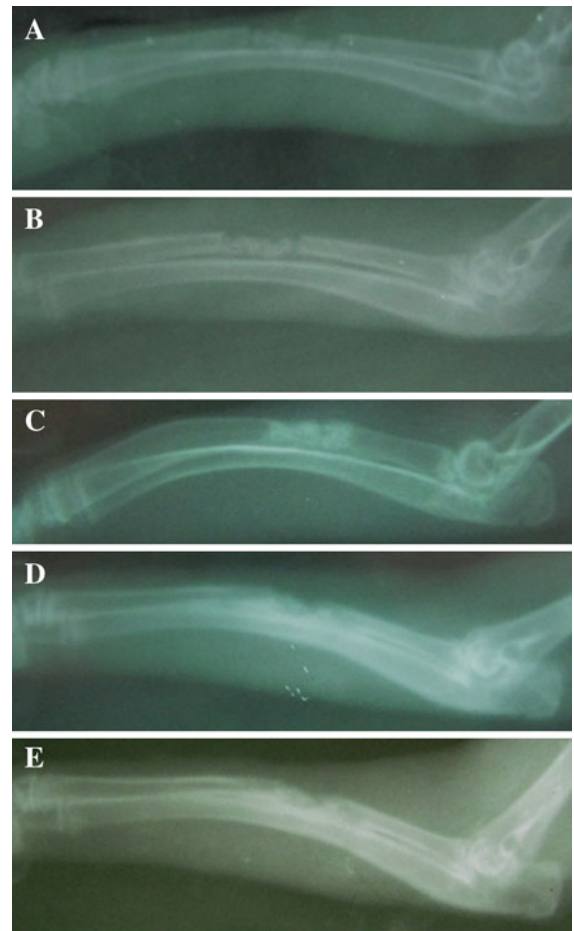


Fig. 3 Radiographs of treated forelimb in hydroxyapatite-hPRP group, on 1st day (a), 14th postoperative day (b), 28th postoperative day (c), 42nd postoperative day (d) and 56th postoperative day (e)

substituted with fibrocartilage tissues. However, the defects of the animals in the hydroxyapatite group showed some angiogenic activities but it was not as well as those of the hydroxyapatite-platelet group (Fig. 4).

Normal trabecular and woven bone were uniformly formed within the defects of the animals that were treated with hydroxyapatite-platelet regimen and the lesions of five from six animals of this group were filled with woven bone and showed proper maturation, however, the defect of the last rabbit contained more fibrocartilage than bone. The regenerated bone completely spanned the defect and most instantly produced full histologic union. Active endochondral ossification and secondary fracture

Table 3 Radiographical findings for proximal union at various post-operative intervals

Postoperative days	Med (min–max)			<i>P</i> ^a
	Control (n = 12)	Hydroxyapatite (n = 12)	hPRP-hydroxyapatite (n = 12)	
14	0 (0–0)	0 (0–0)	0 (0–1)	0.3
28	1 (0–1)	1 (0–1)	1 (1–2) ^{b,c}	0.01
42	1 (0–1)	1 (1–2) ^d	2 (1–2) ^{e,f}	0.00
56	1 (0–2)	2 (1–2) ^g	2 (1–2) ^h	0.01

Significant *P* values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

^b *P* = 0.009 (compared with control by Mann–Whitney U test)

^c *P* = 0.03 (compared with hydroxyapatite by Mann–Whitney U test)

^d *P* = 0.009 (compared with control by Mann–Whitney U test)

^e *P* = 0.01 (compared with control by Mann–Whitney U test)

^f *P* = 0.00 (compared with hydroxyapatite by Mann–Whitney U test)

^g *P* = 0.01 (compared with control by Mann–Whitney U test)

^h *P* = 0.01 (compared with hydroxyapatite by Mann–Whitney U test)

Table 4 Radiographical findings for distal union at various post-operative intervals

Postoperative days	Med (min–max)			<i>P</i> ^a
	Control (n = 12)	Hydroxyapatite (n = 12)	hPRP-hydroxyapatite (n = 12)	
14	0 (0–1)	0 (0–0)	0 (0–1)	0.1
28	1 (0–1)	1 (0–1)	1 (0–2)	0.2
42	2 (0–2)	1 (0–2)	2 (1–2)	0.1
56	2 (0–2)	2 (1–2)	2 (1–2)	0.1

Significant *P* values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

Table 5 Radiographical findings for remodeling at various post-operative intervals

Postoperative days	Med (min–max)			<i>P</i> ^a
	Control (n = 12)	Hydroxyapatite (n = 12)	hPRP-hydroxyapatite (n = 12)	
14	0 (0–0)	0 (0–0)	0 (0–0)	1.00
28	0 (0–0)	0 (0–0)	0 (0–0)	0.3
42	0 (0–0)	0 (0–0)	0 (0–1) ^{b,c}	0.002
56	0 (0–1)	1 (0–2) ^d	1 (0–2) ^e	0.02

Significant *P* values are presented in bold face

^a Kruskal–Wallis non-parametric ANOVA

^b *P* = 0.009 (compared with control by Mann–Whitney U test)

^c *P* = 0.009 (compared with hydroxyapatite by Mann–Whitney U test)

^d *P* = 0.02 (compared with control by Mann–Whitney U test)

^e *P* = 0.02 (compared with control by Mann–Whitney U test)

repair took place in the middle of the defect of the animals of the hydroxyapatite-platelet group (Fig. 4). No significant inflammatory response was evident in

the lesions of the animals of different groups at 8 weeks post injury, although it may have been present earlier (Figs. 5, 6, 7, 8, 9).

Table 6 Bone measurements at macroscopic and microscopic level

Bone type evaluation	Med (min–max)			<i>P</i> ^a
	Control (n = 6)	Hydroxyapatite (n = 6)	hPRP-hydroxyapatite (n = 6)	
Macroscopic union*	1 (1–2)	2 (2–3) ^b	3 (2–3) ^c	0.00
Microscopic evaluation [†]	2 (1–5)	6 (5–7) ^d	7 (6–7) ^e	0.001

Significant *P* values are presented in bold face

* Complete union (+3 score), presence of cartilage, soft tissue or cracks within the defect indicating a possible unstable union (+ 1 or +2 score), complete instability at the defect site indicating nonunion (0 score)

[†] Empty (0 score), fibrous tissue only (1 score), more fibrous tissue than fibrocartilage (2 score), more fibrocartilage than fibrous tissue (3 score), fibrocartilage only (4 score), more fibrocartilage than bone (5 score), more bone than fibrocartilage (6 score) and bone only (7 score)

^a Kruskal-Wallis non-parametric ANOVA

^b *P* = 0.00 (compared with control by Mann–Whitney U test)

^c *P* = 0.00 (compared with control by Mann–Whitney U test)

^d *P* = 0.005 (compared with control by Mann–Whitney U test)

^e *P* = 0.003 (compared with control by Mann–Whitney U test)

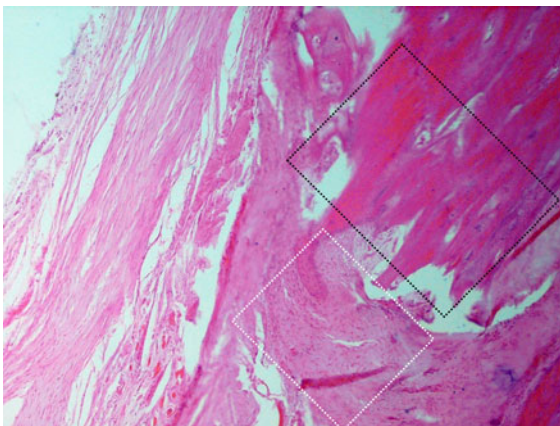


Fig. 4 photomicrograph of control group note to extensive fibrous connective tissues in the defect area without bone marrow formation (*white rectangle*), note to old bone region (*black rectangle*) (H & E stain × 10)

Biomechanical findings

There was statistical significant difference (*P* = 0.01) between the injured bone with normal bone of the control group in terms of ultimate strength and stiffness and the normal bones had superior ultimate strength and stiffness (*P* = 0.04) compared to their normal contralateral bones. However, the ultimate strength and stiffness of the treated animals of both hydroxyapatite and hydroxyapatite-hPRP groups showed more advanced values so that they were not statistically significant with those of their normal

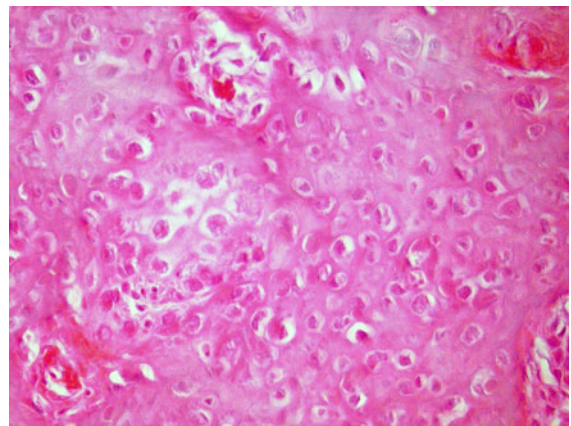


Fig. 5 photomicrograph of control group note to fibrocartilage tissues in the defect area (H & E stain × 40)

contralaterals. There was statistical significant difference between the treated bone of the hydroxyapatite-hPRP with those of the control group (*P* = 0.001) in terms of stress and the treated bones had superior stress compared to those of the injured bones of the animals of the control group (Table 7).

Discussion

To evaluate the bone healing potential of a combination of hydroxyapatite and human PRP a defect model was established in the radial bone of rabbits. This model has previously been reported suitable because

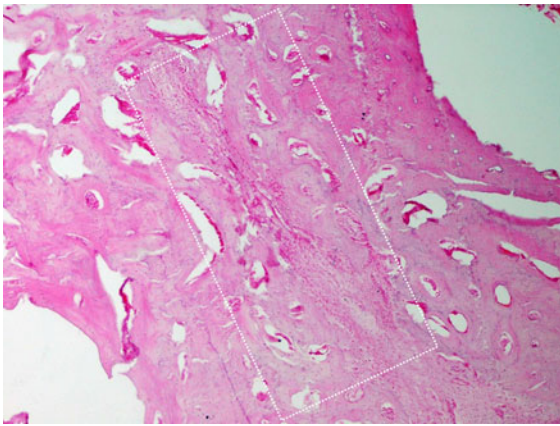


Fig. 6 photomicrograph of hydroxyapatite group, note to the woven bone and fibrocartilage formation (*white rectangle*) and several neovascularisation (H & E stain $\times 10$)

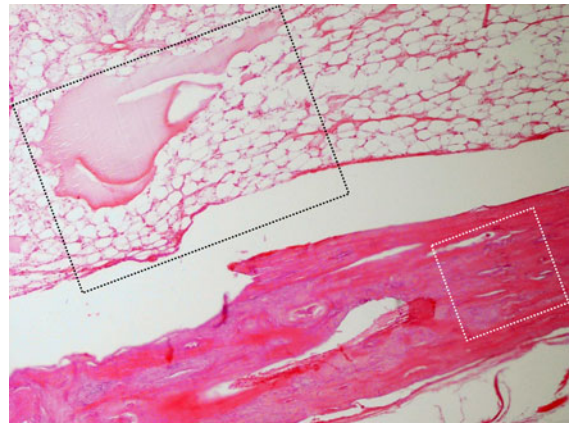


Fig. 8 Photomicrograph of hydroxyapatite-hPRP group, bone marrow formation in grafted area (*black rectangle*) and fusion between hydroxyapatite and old bone (*white rectangle*) (H & E stain $\times 10$)

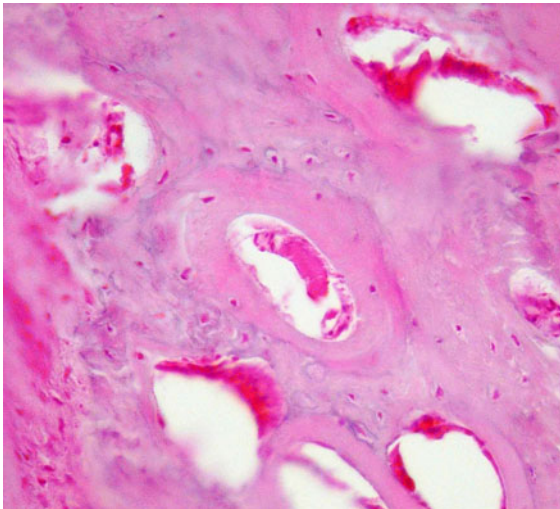


Fig. 7 Photomicrograph of hydroxyapatite group, note to the woven bone (H & E stain $\times 40$)

there is no need for internal or external fixation which influences the healing process (An and Friedman 1999). The segmental defect was created in the middle portion of the radius as long as 10 mm for inducing nonunion defect and to prevent spontaneous and rapid healing (Bolander and Galian 1983).

The hypothesis was on the basis that addition of the hPRP to a mixture of the particulate hydroxyapatite in a critical size defect of the radial bone of rabbit could have a positive effect on bone formation.

The results of the radiological, macroscopical, histological and biomechanical examinations showed that bone healing were enhanced when hPRP was used

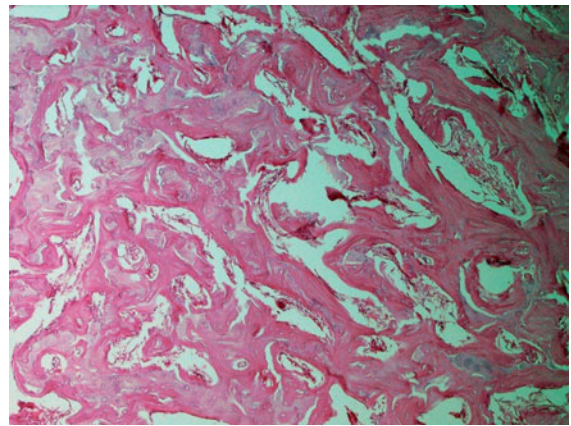


Fig. 9 Photomicrograph of hydroxyapatite-hPRP group, trabecular bone with marrow formation (H & E stain $\times 10$)

concurrent with hydroxyapatite. These results are not in agreement with the rare studies that have been conducted previously (Mooren et al. 2007).

This study was performed to provide an explanation for the existing confusion in the literature regarding the efficacy of PRP treatment in combination with other artificial bone graft substitutes, and to give more insights into the effect of PRP on bone regeneration. To the authors' knowledge this is one of the first studies, which presents new data on the bone regenerative properties of human PRP as a xenogenic PRP concurrent with hydroxyapatite effects on bone healing in rabbit model.

The clinical and experimental data in the literature regarding the osteogenic potential of PRP are

Table 7 Biomechanical findings after 56th postoperative day

Three point bending test criteria	Mean \pm SEM					
	Control (n = 6)		Hydroxyapatite (n = 6)		hPRP-hydroxyapatite (n = 6)	
	Normal limb	Treated limb	Normal limb	Treated limb	Normal limb	Treated limb
Ultimate strength (N)	74.3 \pm 10.0 ^a	38.6 \pm 7.5	60.6 \pm 10.5	70.8 \pm 8.4	108.0 \pm 17.2	95.0 \pm 12.3 ^b
Stress (N/mm ²)	3.64 \pm 0.7	2.18 \pm 0.3	4.1 \pm 0.83	3.75 \pm 0.71	6.5 \pm 0.9	4.3 \pm 0.8 ^c
Stiffness (N/mm)	128.3 \pm 7.4 ^d	91.6 \pm 14.9	96.0 \pm 11.6	75.0 \pm 5.6	76.6 \pm 13.08	83.3 \pm 11.7
Strain (%)	7.9 \pm 0.5	8.4 \pm 0.6	7.1 \pm 1.1	6.7 \pm 0.80	5.8 \pm 0.4	6.6 \pm 0.80

^a $P = 0.01$ (normal limb compared with treated limb in control group by student-*t* test)

^b $P = 0.01$ (treated limb compared with treated limb in control group by one-way ANOVA test)

^c $P = 0.001$ (treated limb compared with treated limb in control group by one-way ANOVA test)

^d $P = 0.04$ (normal limb compared with treated limb in control group by student-*t* test)

controversial. The results of the present investigation confirm a number of clinical and experimental studies demonstrating a positive influence of hPRP on bone regeneration (Marx et al. 1998; Anitua 1999; Schlegel et al. 2004; Thorwarth et al. 2005). However, in human maxillofacial defects, neither autograft nor allograft or a mineral bone substitute material enhanced bone formation when augmented with PRP (Froum et al. 2002; Shanaman et al. 2001; Raghoobar et al. 2005). In a non-critical rabbit skull defect, autogenously PRP was not superior to the empty defect nor did PRP increased bone formation by autogenous bone (Aghaloo et al. 2002).

This study demonstrates the hydroxyapatite-hPRP's role in treating bone defects. From both quantitative and qualitative analyses conducted using the four outcome measurements described in this study, significant differences were present between the defects of the animals of the hydroxyapatite-hPRP treated group and those of the two other groups.

Platelet rich plasma was found to be effective only when it was used together with bone graft. This has been similarly observed in the previous studies (Grageda 2004). Hydroxyapatite as a artificial bone graft was used as it has no osteoinductive potential. Its use with PRP provided the evidence required to demonstrate the tissue-enhancing ability of PRP. Overall, the results of this study correlate with the findings of other studies that supported the use of PRP in almost similar conditions. This has convinced many clinicians and scientists to support its use in clinical practice (Froum et al. 2002; Kim et al. 2002; Marx 2004).

The results of the present study indicate that hPRP in combination with hydroxyapatite stimulates a favorable

reaction in the injured area of the long bones. The radiographic evaluation showed that the bone gap in the hydroxyapatite-hPRP group was healed before that of the control group and it was also already in the remodeling stage. While the defects of the control animals even at the end of 8 weeks post-injury were still in the fibroplasia stage. This fact was corroborated by macroscopic, microscopic and biomechanical data analysis, which showed that osteogenesis in the defects of the animals of hydroxyapatite-hPRP group was stronger than those of the two other groups at 56 days post-injury.

Hydroxyapatite, a crystalline phase of calcium phosphate found naturally in bone minerals, has shown tremendous promise as a graft material. It exhibits initial mechanical rigidity and structure, and demonstrates osteoconductive as well as angiogenic properties in vivo (Kilian et al. 2008; Appleford et al. 2009; Yoshikawa et al. 2000). In osteoperiosteal gaps bridged with hydroxyapatite only, the porosities were invaded with fibrous tissue or fibrocartilage tissues were more than bone tissues. Occasionally, bone formation was observed in direct contact with hydroxyapatite, confirming its osteoconductive ability, but it was insufficient to allow union. These findings are similar to those reported using hydroxyapatite. When the gap reaches a critical size the osteoconductive properties of the material are insufficient to fill the gap with formation of new bone (Ohgushi et al. 1989). Therefore this model proved to be adequate for evaluation of hydroxyapatite as a scaffold for human platelet.

More unexpected is formation of the cortex and medullary canal together with mature lamellar bone

observed in most of the cases. The previous *in vitro* studies have shown that artificial bone graft materials supports the attachment, growth and differentiation of the bone-marrow stromal cells (Petite et al. 1996). The findings of the present study suggest that hydroxyapatite is a suitable resorbable carrier for platelet *in vivo*. It serves as a substrate to promote the stem cells of the bone marrow to attach and grow, and as a template, to guide bone morphogenesis in a clinically relevant volume.

The platelet rich plasma contains several growth factors including isomers of platelet derived growth factor (PDGF), transforming growth factor- α 1 (TGF- α 1), transforming growth factor-2 (TGF-2), Insulin like growth factor-I (IGF-I), Insulin like growth factor-II (IGF-II) and vascular endothelial growth factor (VEGF). All these growth factors are promoters of bone regeneration. Platelet derived growth factor has been shown to be mitogenic for osteoblasts (Assoian et al. 1984) and stimulates migration of the mesenchymal progenitor cells (Fiedler et al. 2002). It has been stated that PDGF was able to induce callus formation in the bone defects of the animal models (Nash et al. 1994). TGF- α also has a stimulative effect on osteogenesis and inhibits bone resorption (Baylink et al. 1993). In addition, it has been reported that IGF-I and the angiogenic factor VEGF induced bone formation in rats (Spencer et al. 1991) and in rabbits (Street et al. 2002), respectively. The findings of the present study suggest that superiority of the angiogenesis in the defects of the animals of group hydroxyapatite-hPRP was possibly due to the presence of VEGF in human platelet. These growth factors support bone regeneration primarily via their chemotactic and mitogenic effects on preosteoblastic and osteoblastic cells. Due to this phenomenon, an enhanced bone formation criteria in the defects of the animals of the hydroxyapatite-hPRP group compared to those of the control ones was observed. However, hPRP does not contain BMPs, the most potent osteoinductive proteins, that are the only growth factors known to induce ectopic bone formation which promote stem cells to differentiate into the osteoblastic lineage (Cook 1999).

The enhanced healing effects of the human PRP after combination with human bone graft material, compared to a combination with a synthetic bone substitute, can also be explained by the mechanism of action of PRP. According to Marx et al. (Marx 2004),

PRP is thought to exert its effects on living cells. Consequently, when PRP is used together with synthetic, non-cellular bone substitutes less promotion of bone formation could be expected compared to its application with the bone graft material. The beneficial effects of PRP applied in combination with a synthetic bone substitute, depend on the number of resident osteoprogenitor cells at the bone defect site. Occasionally, the osteoconductive materials can obscure the true effects of PRP. However, in the present study, combination of hPRP with hydroxyapatite lead to superior bone healing in comparison with the hydroxyapatite alone and the control group. Therefore, based on the findings of the present study, it could be concluded that high concentrations of platelets is effective and lead to superior and faster bone formation. While Schlegel et al. (2004) and Thorwarth et al. (2005) got better results by administering higher doses of hPRP (6.5-fold compared to normal blood) than with lower platelet concentrations (4.1-fold) on bone regeneration in skull defects of minipigs (Schlegel et al. 2004; Thorwarth et al. 2005), some other experimental studies found no correlation between the platelet concentration and the observed biological effects (Aghaloo et al. 2002; Kim et al. 2002).

In overall, possibly one reason in demonstrating such conflicting findings regarding use of the platelet rich plasma in the treatment of a wide variety of “wounds” has to do with the variability in concentrations of growth and differentiation factors in differing preparations of platelet rich plasma. Therefore, an estimate of the concentration of at least one growth and differentiation factor in such hPRP preparations should be performed in the future studies. Unfortunately, in the present study the authors did not quantify either existence or concentration of main growth factors of the hPRP used in their experiment. In fact, it is not the platelet rich plasma, after all, that represents the “healing properties” of what is being used, but rather the combined growth and differentiation factors that the platelet rich plasma presents to some wound site. However, it is suggested in future some of the confusion in the prior literatures.

Conclusions

In conclusion this study demonstrated that hydroxyapatite-hPRP could promote bone regeneration in

critical size defects with a high regenerative capacity. This finding will nominate hydroxyapatite-hPRP as an attractive alternative for reconstruction of the major diaphyseal defects in the long bones in animal models. Combination of functional biomaterials or autografts, precursor cells or osteoinductive growth factors with hydroxyapatite in animal models, in the future studies, may introduce more effective therapeutic regimes in regeneration and bone formation of the long bone injuries.

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