Effects of Concurrent Usage of Human Lyophilized Platelets and Autograft Omentum on Bone Healing in Rabbit Model

Abstract

Introduction: This study was performed to evaluate the effect of lyophilized human platelet rich plasma powder (LhPRP) and omentum autograft on the bone healing properties in animal model which can further be utilized for different orthopedic or maxillofacial reconstructive surgeries.

Materials & Methods: Bone defects of 10 mm diameter were created in diaphysis of radius of 20 rabbits and then either left undisturbed (control group) or filled with one of the following materials: a piece of autogenous omentum, LhPRP, or concurrent use of LhPRP and omentum. Bone formation, union and remodeling, were evaluated at the 2nd, 4th, 6th and 8th weeks postoperatively by radiography, using bone healing criteria. On 56th postoperative day, the operated limbs were removed and further histopathological evaluations were carried out.

Results & Discussion: The results showed that LhPRP with or without omentum in bone regeneration in 10mm defects was significantly superior to control group ($p<0.05$).

Conclusion: LhPRP powder, alone or combined with autogenic omentum, provides increased bone regenerative properties in experimental bone defects in animal model after 56 days.

Keywords: Omentum, Lyophilization, Platelets, Bone regeneration, Rabbits.

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Introduction

Research for substitutions for autogenous bone grafts while enhancing bone healing properties continues. Bone grafts are vastly used in the treatment of nonunion. The ability of these grafts as scaffolds provides enough biomechanical strength for tolerating compressive forces applied to the bones during gait. Bone grafts have a noticeable effect on promoting osteogenesis, which eventually leads to enhancing bone formation[1]. Limitation of availability of graft amount and the donor-site affection in autografts and allografts disease transmission are the most common disadvantages of using of these grafts. In general, there are two groups of bone grafts: The biological-based material, like hydroxyapatite;or the synthetic based material like calcium carbonate- both with their own advantages and disadvantages. The methods of biological substance usage, cell therapies and mechanical induction that contain osteogenic promoters are still novel and expensive; except for one product PRP (platelet- rich plasma)[1]: LhPRP is safe and also easily produced[2, 3]. Formerly, LhPRP has been used for orthopedic bone healing purposes and also in maxillofacial surgeries; however, there have been contradictory outcomes on the benefits of this material, since both good and poor results have been reported[2, 4]. Variable factors can influence clinical studies data: defect size, defect site and patient related factors, whilst the experimental studies that have been done in this field did not clarify if the LhPRP used had comparable concentrations. Hence, it is necessary to study and characterize the true effectiveness of LhPRP in treating nonunions or delayed unions which could lead to justifying its usage in clinical practice

Materials &Methods

Animals

In this study, twenty white New Zealand adult male rabbits, 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 divided into 4 groups (n=5) randomly: control group, omentum group, LhPRP (Human platelet was prepared from the Blood Transfusion Organization) group and omentum-LhPRP group. All the animals underwent subcutaneous injection of anthelmintic drugs and were fed normal diet and hospitalized from 15 days before surgery and let to move freely. The ethics committee of the university approved the study according to animal rights protocols during the study.

Preparation of lyophilized platelet

Human PRP was prepared and supplied by the Shahrekord 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 22^0C. 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 × 10^9 /l and 2,422 × 10^9 /l respectively. The fresh bag of platelets was transferred to the laboratory and lyophilized at a temperature of 22°C according to previous study[1]. Finally, the lyophilized platelet was storage frozen until surgery.

Surgical Procedure

Right forelimbs of the animals were shaved and surgically prepared. All animals were anesthetized by injection of Ketamine (30mg/kg) and Acepromazine (0/2 mg/kg) and maintained with Isoflurane with anesthesia machine.

From a craniomedial approach, bone defects were created in critical size (10 mm) in the radial bone diaphysis of all rabbits and then filled with a piece of autogenous omentum harvested from abdomen (treatment group), LhPRP (0.5 ml of prepared lyophilized PRP was used in defected area in treatment group), concurrent use of LhPRP (0.5 ml) and autogenous omentum (treatment group) or the defect left empty as control group. Surgical site was closed with 2-0 absorbable suture material (polyglactin 910) in 2 layers. To harvest autogenous omentum, a ventral midline approach was used, omentum was harvested aseptically and linea alba closed routinely. The animals were hospitalized without any external fixation. Antibiotic therapy was done with Penicillin (40000 IU/kg IM) and Streptomycin (12mg/kg IM) post-operatively for 2 days. The animals were monitored daily to obtain information about weight bearing properties and wound condition.

Radiographic Evaluation

Lateral radiographs (45kv/ 20mAs) were taken postoperatively at the 2nd, 4th, 6th and 8th weeks and bone formation, union and remodeling of the defect were evaluated by modified Sandhu & Lane scoring system (Table 1)[2].

Radiological criteria such as bone formation, proximal union, distal union and remodeling summation were calculated and compared between groups blindly by two radiologists in mentioned intervals.

Histopathologic Evaluation

All animals sedated with using intramuscular injection ofXylazine (5 mg/kg) and ketamine hydrochloride (40 mg/kg) and then were euthanatized using Mg Sulfate solution injected directly into the heart on 56th postoperative day[3]. All operated bones were removed, prepared and stained with hematoxylin and eosin, blindly scored by pathologist using Emery's scoring system includes: gap remained vacant (score = 0), gap occupied with only connective tissue (score = 1), gap filled with more fibrous tissue rather than cartilage (score = 2), more cartilage than fibrous tissue (score = 3), only cartilage (score = 4), more cartilage than bone (score = 5), more bone than cartilage (score = 6), and filled with bone only (score = 7)[4].

Statistical Analyses

First of all, data were compared by Kruskal-Wallis, non-parametric ANOVA. When *p-values* were less than 0.05, the pair wise group comparisons were performed by Mann-Whiney U test (SPSS version 26 for windows, SPSS Inc, Chicago, USA).

Results

During the study all of animals were alive without any suffering or complication.

Radiographic findings

There was a significant difference in bone formation, union and remodeling criteria of the bones between control and LhPRP group ($p<0.05$) on 6th week and on 8th week ($p<0.02$) that LhPRP group was superior to control group. On 8th week, there was a significant difference between omentum and LhPRP groups ($p<0.02$), with LhPRP group showing better results (Table 2, Figure 1-4).

Histopathological findings

Histopathologically, there was not any signs of inflammation or infection after 56 days. Regarding bone formation, union and remodeling, there was a significant statistical difference between control group with omentum group ($p = 0.02$), with LhPRP group ($p = 0.05$) and also with LhPRP-Omentum group ($p = 0.02$); healing was shown to be drastically slower in control group in comparison to the other three groups (Table 3, Figure 5-8).

Table 1: Lane & Sandhu scoring system

Grade	Bone Formation
0	No bone formation
1	Bone formation filling 25% of the gap
2	Bone formation filling 50% of the gap
3	Bone formation filling 75% of the gap
4	Bone formation filling 100% of the gap
Grade	Bone Union (proximal and distal union separately)
0	No union
1	Possible union
2	Complete union
Grade	Remodeling
0	No remodeling observed
1	Remodeling of the medullary canal
2	Complete remodeling of cortex
Grade	Total points possible per category
4	Bone formation
2	Proximal union
2	Distal union
2	Remodeling
10	Maximum Score

Table 2: Radiological findings for bone defect healing (scores summation) at different postoperative intervals.

Postoperative weeks	Med (min-max)				*P* [a]
	Control	Autogenous Omentum	LhPRP	LhPRP-Omentum	
2nd	3(3-6)	3(2-8)	4(1-4)	3(1-5)	0.2
4th	3(3-9)	4(4-9)	6(6-9)	5(5-9)	0.8
6th	3(3-9)[b]	6(6-10)	9(8-10)	6(6-9)	0.2
8th	4(4-10)[c]	7(7-10)[d]	10(10-10)	10(8-10)	0.05

[a] Kruskal-Wallis test was performed. P values less than 0.05 were considered significant. Mann-Whithney U test was subsequently performed.

[b] Statistically significant difference observed between control and LhPRP groups ($p<0.05$) in 6th week. LhPRP group showing better results.

[c] Statistically significant difference observed between control and LhPRP groups ($p<0.02$) in 8th week.

[d] Statistically significant difference observed between omentum and LhPRP groups ($p<0.02$) in 8th week.

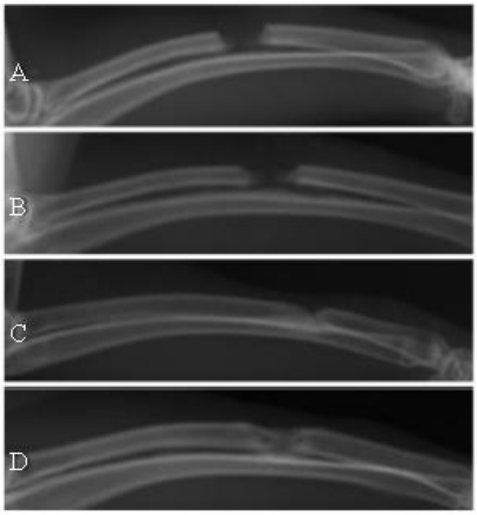

Figure 1: A: Radiograph of the control group, 2nd postoperative week. B: Radiograph of the control group, 4th postoperative week. C: Radiograph of the control group, 6th postoperative week. D: Radiograph of the control group, 8th postoperative week.

Figure 2: A: Radiograph of the omentum group, 2nd postoperative week. B: Radiograph of the omentum group, 4th postoperative week. C: Radiograph of the omentum group, 6th postoperative week. D: Radiograph of the omentum group, 8th postoperative week.

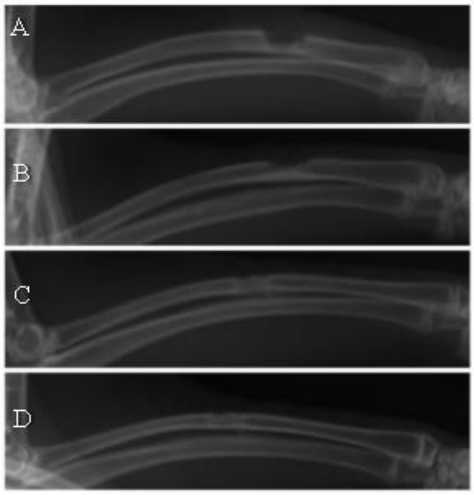

Figure 3: A: Radiograph of the LhPRP group, 2nd postoperative week. B: Radiograph of the LhPRP group, 4th postoperative week. C: Radiograph of the LhPRP group, 6th postoperative week. D: Radiograph of the LhPRP group, 8th postoperative week.

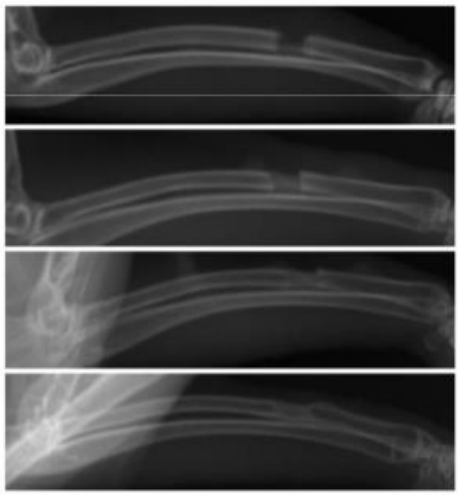

Figure 4: A: Radiograph of the LhPRP-Omentum group, 2nd postoperative week. B: Radiograph of the LhPRP-Omentum group, 4th postoperative week. C: Radiograph of the LhPRP-Omentum group, 6th postoperative week. D: Radiograph of the LhPRP-Omentum group, 8th postoperative week.

Table 3: Histopathologic evaluation of bone defect healing.

	Control	Omentum	LhPRP	LhPRP-Omentum	P [a]
Histopathologic Evaluation	3(3-6)[b]	7(7-7)	6(5-7)	7(6-7)	0.02

[a] Kruskal-Wallis test was done. P values less than 0.05 were considered significant.

[b] Significant statistical differences between control group with omentum group (p = 0.02), with LhPRP group (p = 0.05) and also with LhPRP-Omentum group (p = 0.02) were found; healing was shown to be drastically slower in control group.

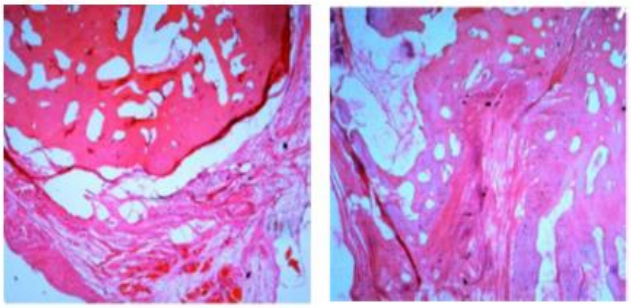

Figure 5: A: Fibrous plaque occluding the bone. B: Fibrous plaque surrounded with trabecular bone. 10X (H&E).

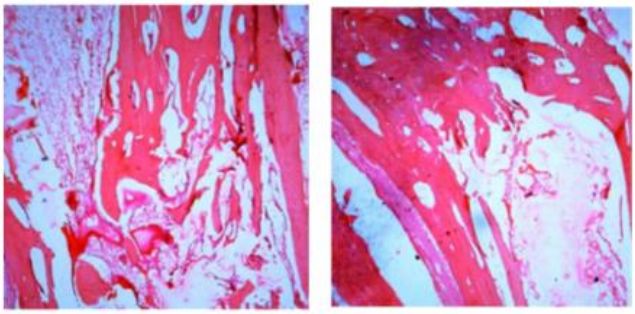

Figure 6: A: The defect area is delineated. B: Defect healing with trabecular bone. 10X (H&E).

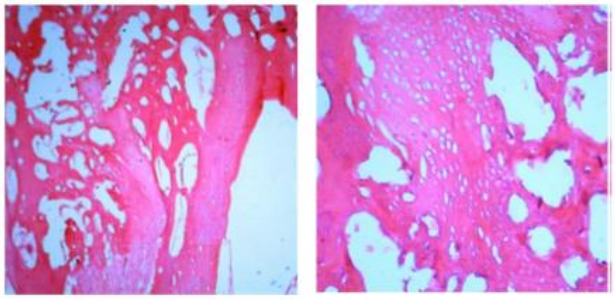

Figure 7: A: Trabecular bone presence and bone marrow formation. B: Hypertrophic cartilage and presence of chondrocytes. 10X (H&E).

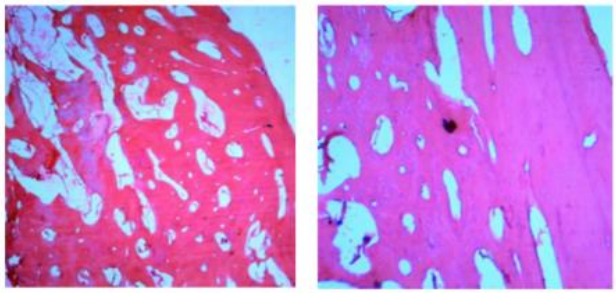

Figure 8: A: Bone marrow is remodeling. B: Hypertrophic cartilage; presence of bone and cartilage. 10X (H&E).

Discussion

The main motivation of the current study was to add more clarity regarding the usage and effectiveness of LhPRP, also providing additional insight into the properties of LhPRP, omentum and their combination on bone regeneration.

As it has been indicated before, this kind of defect in rabbits does not necessarily need any internal or external fixation method to achieve healing, hence it has been reported suitable for bone reconstructive surgeries[5]. In order not to allow spontaneous rapid healing, the gap created in the mid radii was as wide as 10 millimeters[5].

According to the currently controversial data about the efficacy of LhPRP, its osteogenic properties are yet to be the subject of further researches. As the results of the current research indicate, it could be implicated that LhPRP exploitation might effectively enhance bone regeneration properties[6, 7]. However, utilizing LhPRP along with autograft, allograft or mineral bone substitute material did not show any increase in bone regeneration process[8, 9]. Another study showed that LhPRP lead to inferior bone formation in rabbit skull defect, whether used alone or along with autogenous bone[10].

Based on the results of the current research, LhPRP is a significant stimulant for bone regeneration in defective areas. On the 2nd post-operative week radiological evidences indicate that in the LhPRP group, the defective area is in remodeling phase and the healing of the bone has been faster in comparison with control group. In addition, 8 weeks in the control group, the healing stage is insufficient; histopathologic data analysis also confirms this phenomena. The analysis has shown that osteogenic process in the LhPRP group at 56th post-operative day was more advanced compared with the control group.

Different types of growth factors such as isomers of PDGF, TGF-X 1, TGF-X 2, IGF-I, IGF-II and VEGF presence in LhPRP that could lead to enhance of bone regeneration. Also PDGF has mitogenic characteristics for osteoblasts[11] and is also stimulant of the mesenchymal progenitor cells migration[12]. Callus formation properties ofPDGF in bone defectshas been approved previously in animal models[13]. TGF-X also has osteogenesis properties with inhibition of bone resorption[14]. Further reports

have shown that IGF-I and VEGF are capable of enhancing bone regeneration in laboratory animals[15, 16]. This actually seems to be the process responsible for the better and boosted bone regeneration in the LhPRP group in comparison with control group. Although LhPRP does not contain BMPs; they are known as proteins with the most osteoinductive potency resulting in osteoblastic differentiation from stem cells, also having the ability for induction of ectopic bone formation[17]. It is stated that administering higher doses of LhPRPhas led to better results in bone formation of minipigs defective skulls comparing to lower platelet concentrations[18, 19]. Although other experimental researches do not confirm a connection between the platelet concentration and bone healing enhancement[10, 20].

Since omentum benefits from a proper vascularization, it contains significant amounts of nutrients, oxygen, and angiogenic and growth factors, and being grafted can provide an appropriate condition for bone formation[21]. Augmentation of oxygen concentrations depends on an adequate vascular flow which can lead to induction of the osteoprogenitor cells production from the perivascular mesenchymal cells[22]. Whilst the angiogenesis is happening, VEGF causes the increased capillary permeability[23], it also provides high levels of oxygen concentration, both of which being the possible effective factors of enhanced osteogenesis in defective bones[21]. The process mentioned above which induces bony tissues was observed more advancely in the omental group compared with control group, which is probably related to fewer amount of blood vessels in the control group's defective area compared with the other group. Also in the process of tissue regeneration, sufficient amount of available oxygen in the tissue is crucial since it improves phagocytosis and could prevent infection[24]. This criterion is also considered indicative of omentum exploitation as autogenous grafts. Accordingly, omentum releases angiogenic and growth factors which further activate macrophages, and this process eventually leads to new capillaries sprouting and invading the fibrous tissue[24].Karim et al. have also shown enhanced healing in the omental-coral groups at radiological and histopathological evaluation at 60th post-operative day[25].

Bigham et al. have proven that omental-culture medium and omental-ASCs groups have better osteogenic potential in healing of the radial bone defect[26]. In the present study, concurrent use of omentum with LhPRP promoted better bone regeneration than that of the control group.

The radiological, and histopathological findings of the present study have shown that the reparation of the defect in the control group was not as efficient and the defective gap was mostly occupied with fibrous tissues and rarely with cartilage instead of osseous tissue. Barnes et al. demonstrated that the mesenchymal derived chondrocytes proliferate and develop into cartilaginous matrix. Separate cartilaginous cores grow and merge to produce a central fibrocartilaginous plug between the fractured fragments that splints the fracture[27].

Unlike LhPRP derived from human blood, Preparation of lyophilized PRP (LPRP) from the animal blood is not a standardized procedure hence, it was impossible to provide LPRP derived from rabbit blood from the relevant centers. Preparing the rabbit LPRP from the same animals needed a great volume of blood to be obtained from each rabbit and the Ethics Committee laws did not allow such a procedure and also believed this might affect their regenerative capacity. There is still a lot to be defined about LhPRP; the critical efficent amounts of platelets in LPRP for different animal species, levels of growth factors in different animal species and similarities or differences in their mechanisms of action. Until then, the animal LPRP preparations and studies must continue carefully[28].

The production of reactive antibodies against LhPRP must be considered, since this process might also affect the results, however, in the present study, no histological evidences of acute or chronic inflammatory response to LhPRP xenograft was observed, although it may have been present earlier.

One of the main motivations of this study was that LhPRP contents capacity to enhance healing have been proved in earlier studies, however, the rabbit's LPRP contents have still been unknown for science society.

Conclusion

Regarding the results, it seems that LhPRP powder alone and with the usage of autogenic omentum provides increased bone regenerative properties in experimental bone defects in animal model after 56 days only in radiological and histopathological evaluations.

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