Radiographic & histopathological analysis in calvarias bone regeneration process by platelet-rich plasma, platelet-rich plasma–gel And auto bone chips in rat

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ABSTRACT

A functional treatment for skeletal damages in orthopedic and oral maxillofacial surgeries is required. Platelet growth factors such as Platelet Derived Growth Factor (PDGF), Bone Morphogenic Factor (BMP), Transferring Growth Factor-β (TGF-β) and Insulin-like Growth Factor-1 (IGF-1) proceed wound healing and bone regeneration. In the present study we focused on the effect of platelet rich plasma (PRP), platelet rich plasma gel (PRP-Gel) and auto bone chips on this process. 30 male, 22 weeks old, Sprague-Dawley rats weighing 525 g were used. They were divided in three groups consisting of PRP (treated by Platelet-Rich Plasma), PRP-Gel (treated by it), Bone chips and Control (two cavities created in each animal in this group). After 16 weeks they were histologically investigated while in the periods of 40, 60, 90and 120 days, the radiography had been done. The radiographic analysis showed complete treatment in all groups; however, by the histo-pathological investigations by auto bone chips complete and PRP-Gel partial healing has been observed. By histo-morphometric surveys (100±25) % in bone chips and (50±25) % in PRP-Gel groups bone bridging were observed, whereas in PRP it was not noticeable. The Present study suggests that neither PRP, nor PRP-Gel could be as beneficial as bone chips. Statistically, in PRP-Gel group, due to the existence of fibrin and thrombin, solid bone bridging at the treated site is indicated. According to the previous studies, in which the key role of both inhibitory and stimulatory signals in controlling the bone regeneration were proven, we suggest that auto bone chips could completely enhance healing due to signals among blood factors, environmental tissues and skeletal particles.

Keywords: PRP; PRP-Gel; Bone chips; Calvarias

INTRODUCTION

Skeletal damages and disorders would be caused by various agents and commonly take place in any parts of the body. However, previous studies have introduced several solutions, but the requirement for a suitable graft to provide bone regeneration specifically in functional treatments in orthopedic and oral maxillofacial, seems to be needed [1]. Therefore, the significance of mechanical and biochemical factors is distinguished [2]. Since 1990, medical science has found several components in blood which could accelerate the healing process. In 1994 Tayapongsak et al used autologous fibrin adhesive (AFA) during mandible continuity reconstruction. Then, they attributed to enhance osteo-conduction which is given to osteo-compotent cells in the graft by the fibrin network that is developed by the AFA [5]. Platelet-Rich Plasma was first described for use with mandible grafts by Marx et al in 1998. They suggested that PRP enhanced the healing of autologous bone grafts and attributed this to the concentration of growth factors released by activated platelets. It has been reported that PRP preparation may increase the concentration of platelets up to 338 %. Also, there is increasing evidence that growth factors such as Bone Morphogenic Factor (BMP), Platelet Derived Growth Factor (PDGF), Transferring Growth Factor-β(TGF-β) and Insulin-like Growth Factor-1(IGF-1) [5,6.7], which are secreted from platelet and would initiate the healing process, play a key role in bone regeneration. It has been known that PDGF may affect wound healing including bone as it is synthesized and secreted by platelets, macrophages and endothelial cells. TGF-β participates in the inhibition of osteoclastic activity by stimulating chemotactic migration

of osteoblasts to the site of injury. It is recently been hypothesized that due to the co-linearity between growth factors and bone regeneration, PRP, PRP-Gel and auto bone chips could have an important function in this process, while they are full of fresh concentrated platelet [8,9,10]. The objectives of the present study were to evaluate the osteo-conductive potential of PRP and PRP-Gel and auto bone chips on bone regeneration using a critical-size rat calvarias defect model. Alternatively, this study suggests that neither PRP nor PRP-Gel is as effective as the auto bone chips, which is withdrawn from the rat Calvarias. It proposes the growth factors are not the only elements in bone regeneration; perhaps the condition of the recipient site and each individual in addition to the individual bone character are considerable. Actually, we should notice that the release of growth factors takes place under specific circumstances, in which stimulatory and inhibitory factors regularly affect wound healing, and providing these conditions is more important than the existence of the full growth factor platelet or platelet gel externally.

MATERIALS AND METHODS

The present experiment was done on 30 male, 22 weeks old, Sprague-Dawley rats weighing 545 g. The rats were divided into three groups of ten animals. In each group a cavity was created by trephine drill in the Calvarias 6.2 mm in diameter. Group1 (treated by PRP/Bio-Oss) and group 2(treated by PRP-Gel /Bio-Oss), while group 3 had two cavities; one as a control and the other received Bone chips mixed with individual blood. They were investigated in the periods of 30, 60, 90 and 120 days and radiography on Calvarias was done. After16 weeks they were sacrificed and then histopathology analysis was done.

PRP and PRP-Gel Preparation

PRP was obtained and prepared during surgery from autologous blood using autologous platelet concentrated system. Firstly, 4-5 ml blood was withdrawn from central vein and added to the Acid-Citrate-Dextrose(ACD) covered chamber and centrifuged in 5600 rpm, by which the blood was separated in three layers consisting of Platelet-Poor Plasma, Platelet-Rich Plasma and RBC. PRP was added to calcium chloride that neutralized anticoagulant effect of ACD, and bovine thrombin, which initiated clotting process. After 6 min the PRP-Gel was appropriate to use.

Bone chips preparation

Following anesthetization of Sprague-Dawley rats weighing 545 gr by Ketamine (20mg/kg) and Aspromizine (0.2mg/kg)a piece of calvarias bone in size 6.2 mm in diameter was obtained. The cortical shafts were cleaned with several rinses of PBS and soaked in ethanol to partially remove lipids and cellular debris. After freeze-drying, they were further ground into particle sizes of 100-500 um, and decalcified with 0.6 N HCl/1% Triton X-100.

Radiography analysis

Rat calvarias samples were separated and radiography by X-ray was done. They were noticed by dentistry analyzer and converted to the digital pictures .Differentiation and differences between mineralized and un-mineralized tissues were noted. Based on our calibration the diameters of the cavities were measured during that period and blindly three experts investigated the radiography pictures.

Histopathological investigation

The tissue samples after normal and routine processing were embedded in paraffin wax. Slices of 4 µm thick were prepared and stained with Hematoxylin and Eosin (H & E) for evaluation with light microscopy. Morphological evaluation of the calvarias tissue was done blindly. In consistency with the histo-pathological investigation, histo-morphometric analysis was done.

Histomorphometric analysis

In this method the length of each section according to the first diameter and after calibration with a ruler and pixel was measured and photography was done. The mount of bone regeneration has been calculated by measuring the width of bone filled defect. Examiner for the histomorphometric analysis was evaluated by calculating the correlation coefficient for repeated measurement ($r = 1.0$; $P<0.001$). A defect was considered exhibiting limited bone fill when less than 25 % of the defect width was filled with bone. Partial bone filling was considered when it was more than 25% and less than 90% and complete bone fill was occurred when more than 90 % of the defect width was filled with bone.

RESULTS

In the histopathological surveys, significant results were observed. In the PRP group the healing was not statistically noticeable ($P<0.05$), moreover in histopathological analysis 70 % of samples showed only vascular fibrous union and did not show solid bone bridging on gross examination(Figure1and 2) whereas solid bone bridging and regeneration was observed in PRP-Gel treated site ($P<0.05$) (Figure 3).

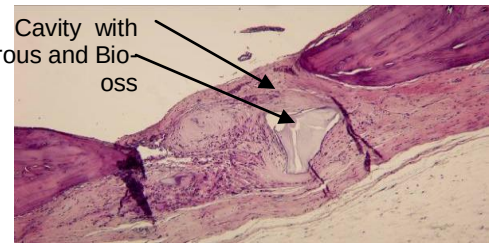


Figure 1. a-Vascular fibrous tissue with inflammatory cells in PRP group after 120 days in calvarias defect (E&H, 10X)

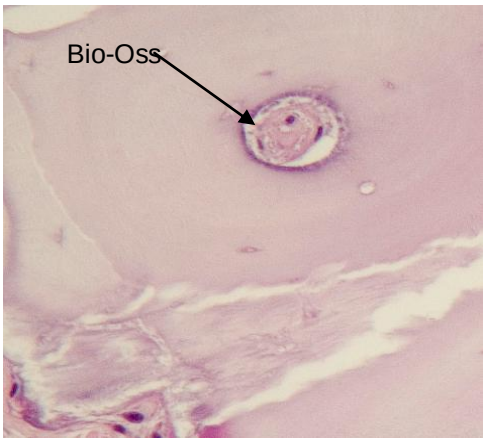
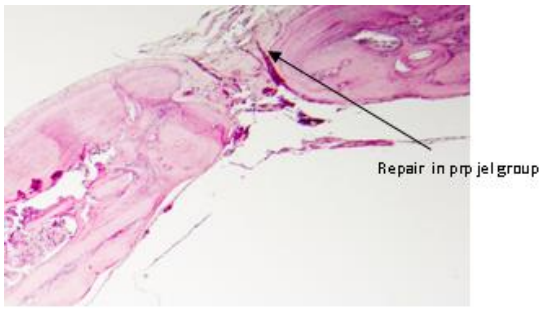


Figure 2. Bio-Oss particle without osteoblast cells (After 60 days E&H 40X)

The bone chips group showed extensive bone formation throughout the defect ($P<0.05$) (Figure 4). In contrast, the PRP group showed large islands of either fibrous tissue or residual non vital bone particles in the defects. In both PRP-Gel and Bone chips groups the surface of new bone was dressed with osteoblasts, indicating active bone formation.

The results of the histomorphometric analysis for PRP, PRP-Gel and auto bone chips are shown in table and graph 1. Percentage calculation for areas showing bone regeneration within the former defect outline were (25±25) % for PRP, (75±25) % for PRP-Gel and (100±25) % for Bone chips group. Taken together, the differences between these groups were statistically significant and suggest the best bone regeneration in the auto bone chips group (Table 1). In the radiography investigation which was done periodically in the days 40, 60, 90, and 120, gradual healing in all groups was seen.



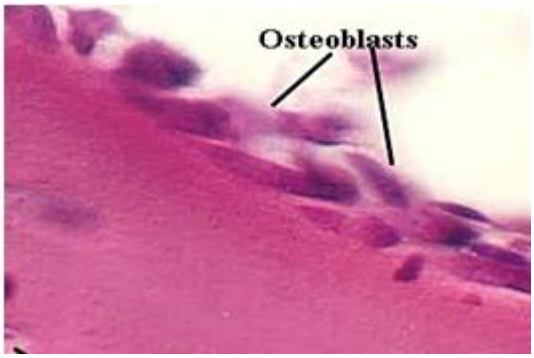


Figure 3. Partial healing in PRP-Gel group / the figure of Osteblast after 120 days in calvarias bone (E&H 10X / 40X)

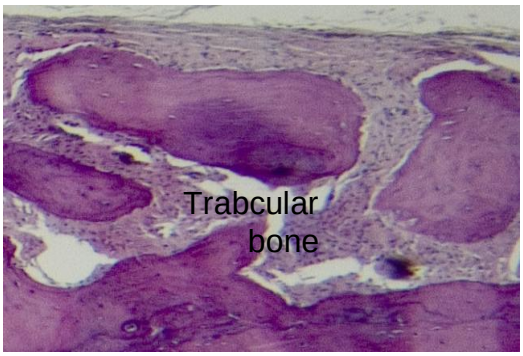


Figure 4. Total healing in calvarias in Bone chips group after 120 days (E&H 40X)

Table 1. The rate of Bone regeneration and its percentage based on Histomorphometric analysis ($p<0.05$). [1=Bone chips, 2=Control, 3=PRP-Gel, 4=PRP].

Group	No.	Mean	SD	Min	Max
1	10	5.89	0.65	4.65	6.20
2	10	2.17	1.08	1.55	4.66
3	10	4. 50	1.14	3.10	6.20
4	10	0.78	1.32	0.00	3.10
Total	40	3.33	2.26	0.00	6.20

ANOVA Analysis

Bone Analysis	Sig
Between groups	P<0.05
Within each group	P>0.05

The results given by three experts, propose that these surveys in bone regeneration are not as reliable as histopathological analysis (Table 2). When the calvarias were relatively healed, the radiographies showed complete healing and they were not coordinated with histopathological analysis (Figure 5). The rate of Bone regeneration in Radiography analysis done by negatoscope (X ray illuminator) and image tools computer software. The widths of bone filled defect were analyzed during the periods. (For Radiography analysis Bone cavity's size was 0 to 4.65 measured by pixel /diameter). **(**Table 2**)**

Table2: Radigraphic results

Group / Day	1	2	3	4
0	0.00	0.00	0.00	0.00
40	1.55	1.55	1.55	1.55
60	3.10	3.10	3.10	3.10
90	3.56	3.56	3.56	3.56
120	4.65	4.65	4.65	4.65

Some studies showed considerable increase in the bone size, which is treated by growth factor in experimental group in comparison with the control in skeletal disorders by tomography micro computer. Interestingly, radiography and histopathological surveys should be done together. The ANOVA test was done while this supposition was tested by Bartlett and Turkey Kramer ($p<0.05$). In bone chips group the most amount of bone regeneration was observed. Then in PRP-Gel group the partial bone regeneration took place in PRP group the mount of regeneration was even less than control.

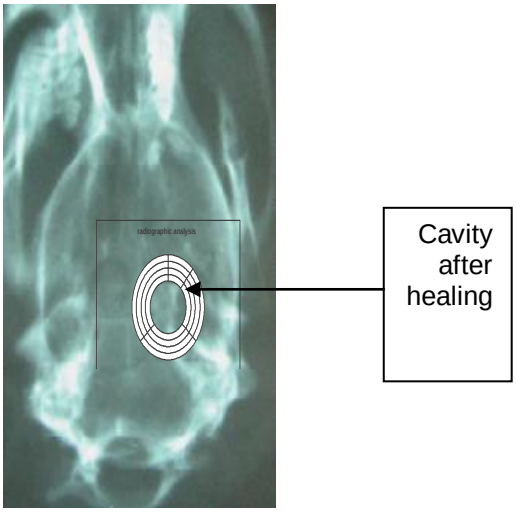


Figure 5. Radiography schema of the damaged region in calvarias (Special tool for measurement of cavity in rat's calvaria)

DISCUSSION

Bone healing and regeneration is an important process in bone graft and according to its side effects , the use of more studies leads to new techniques such as cultured osteoblasts, stem cells or frozen-dried bone in addition to growth factors or connectors with collagen and other membranes that accelerate and promote regeneration seems to be useful . In the present it has been attempted to introduce the new technique in order to achieve this goal. As it had previously been noted, Platelets contain growth factors such as PDGF, TGF-β, IGF-1 have significant effect on wound healing and bone regeneration [3]. Our results showed this potential of growth factors in the PRP and PRP-Gel groups, where the existence of them could almost accelerate the bone regeneration. Although many studies based on these theories, our study suggest that releasing these growth factors needs special circumstances and specific signals, while for each of them, besides stimulator, there are inhibitors too. Reportedly, PRP is a useful agent in the bone regeneration process, while it consists of fresh, proliferated,

concentrated platelet and as it is known, platelet secretes growth factors that proceed bone regeneration. However, various studies noted this effect by the histo-pathological techniques [14], there are some experiments that refuse the relationship between PRP and bone regeneration [17]. It could be noticed that due to the different conditions in each experiment, various results are unavoidable. Nevertheless, the use of collagen type 1 with PRP, because of its potential in growth factors transportation, would possibly be noticeable. As it has previously been reported, PRP at the certain concentrations may inhibit bone regeneration. With respect to the biological effect of PRP on bone regeneration in a graft, the present results contradict with the findings of others, who found that the combination of PRP and Bio-Oss can increase the rate of osteo-genesis and enhance bone formation. In the present study it has been found not any significant effect of PRP on bone formation, which could be attributed to the PRP concentration or the inhibitory signals sent by growth factors. It is not quite clear why the PRP treated bone injury exhibited decreased bone formation as compared with other groups. Thus, more research into the optimal concentration of PRP is necessary.

There is increasing recognition that PRP-Gel is a suitable material used in bone healing process, because it is compressed by concentrated growth factors. It has been reported that fibrin has homeostatic effects and promote wound healing, as it stimulates neovascularization of bone with accelerated healing and earlier new bone formation. But it is suggested the activity of inhibitors in platelet could influence the stimulatory factors in bone regeneration and inactivate them. Thus, the healing process in PRP treated samples was considerable. Although in the present study, the PRP-Gel group, due to the existence of plasma and fibrin, could provide the suitable scaffold and induce healing process. Though, deletion and addition of some materials for preparation could influence the bone regeneration , it has not been distinguished, when the peak time of stimulators and inhibitors activity is, even though their effect on osteoblasts on each scaffold in vitro would be noticeable for clinical use which had been also proposed by previous studies.

In the bone chips group, complete bone healing seems to be the result of sufficient material for this process and the appropriate signals among blood factors, environmental tissues and the skeletal particles. Consistent with the present study, growth factors are not the only agent for bone regeneration. Alternatively, signal pathways that express the specific genes and activate them, show similar trends. Therefore, complemented studies follow signals which inhibit or stimulate this process, seems to be significant. The main point in the present study is blood protein in peripheral blood system and the specific cells which by fantastic balance could transfer the materials to injury side and induce special signals in this process and less is known about them. We suggest that use of patients' autogenous bone chips is more beneficial, because providing blood samples for PRP and PRP-Gel would put them in trouble and takes a long time and expenses.

In the present study it has been tried to study the effect of PRP, PRP-Gel and auto bone chips in promoting bone regeneration in calvarias defect. Our study demonstrated that the auto bone chips in compare with PRP and PRP-Gel could more considerably accelerate and promote bone regeneration. Thus in future it could be suitable method in orthopedic surgery in order to reduce the side effects.

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