Platelet-rich plasma (PRP) enhances bone healing in non-united critical-sized defects: A preliminary study involving rabbit models

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Platelet-rich plasma (PRP) enhances bone healing in non-united critical-sized defects: A preliminary study involving rabbit models


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A B S T R A C T

Introduction: The use of bone grafts in treating non- or delayed unions as the result of large bone loss is well established. However, despite good outcomes, the time to achieve complete union is still considerably long. To overcome this problem, the use of platelet-rich plasma (PRP) has been advocated albeit with varying success. To determine the true effectiveness of PRP in treating non-/delayed unions, a study was conducted using (n = 12) rabbit models.

Methods and materials: Critical-sized defects measuring 2 cm created in the midshaft of the right rabbit tibias were stabilised using 2.7-mm small fragment plates. A spacer placed in the defects to create a delay in bone union was replaced at 3 weeks with artificial bone grafts (Coragraft™), with or without PRP. The operated limbs were radiographed following the defect creation and at 3, 7 and 11 weeks (at sacrifice). Bone healing and histological changes were later assessed and scored using the appropriate grading systems. Four groups were compared for quality of healing: (group-A) control group, that is, no PRP or Coragraft; (group-B) PRP; (group-C) Coragraft; and (group-D) PRP and Coragraft.

Results: Group-D demonstrated the best bone healing based on radiological, histological and gross findings (Kruskall–Wallis: p < 0.05). Group-C had significantly higher scores than group-B, whilst group-A had significantly lower scores than all other groups (Mann–Whitney U: p < 0.05).

Conclusion: The use of PRP with bone graft significantly improves the quality of bone healing. However, the use of PRP without bone substitute does not provide adequate repair tissue and, therefore, provides little benefit when used independently.

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Introduction

Fracture of the tibia is commonly seen in patients involved in motor vehicle accidents. Although complete healing occurs in the majority of cases, a delay or a complete arrest of bony union has been reported in previous literatures. The treatment of non- or delayed union is time-consuming and will require multiple surgeries. This would, in turn, prolong rehabilitation. This results in longer recovery periods, increased financial expenditures and loss of productivity.

In managing patients suspected of delayed union or nonunion of the tibia, surgeons need to overcome two basic issues: (1) the mechanical stability of the fracture fixation and (2) the biological aspect of fracture healing, which involves the processes of osteogenesis, osteoconduction and osteoinduction. For all these biological processes of bone healing to occur, revascularisation through angiogenesis into the injured tissue is vital. Angiogenesis has been shown by J.H. Holstein to be very active during the process of bone healing, in his calvarial defect model. Other investigators have also shown that bone healing can be enhanced by the use of angiogenesis-promoting agents and retarded by angiogenesis-inhibiting agents.

The use of bone grafts in the management of these cases is well accepted. These grafts act as scaffolds, which provide the necessary biomechanical strength that is required to withstand the compressive forces involved during motion. They also promote the ingrowth of cells and other biological products, which eventually leads to the replacement of these grafts by bioactive tissues. Autologous and allogenic bone grafts are commonly used to treat these conditions. However, the limited availability of graft sites and the donor–site morbidity associated with the use of autologous bone grafts have been a major concern. In allogeneic bone grafts, concerns over transmissible diseases and risk of contamination remain high, making its use less appealing for patients. Bone grafts are generally made from either biological materials, for example, hydroxyapatite or synthetic materials, for example, calcium carbonate. Each of these grafts have unique advantages when used in patients; however, the common deficiency seen in these grafts appears to be in their ability to
promote early bone incorporation, which, in turn, translates to late healing.

The use of osteogenic promoters includes biological substances, cell therapies and mechanical induction. These therapies are still novel and expensive with the exception of one product: platelet-rich plasma (PRP). PRP is not only easy to obtain and produce, but also safe. In many literatures, PRP has been used to improve bone healing in the fields of orthopaedic and maxillofacial surgeries; however, reports on the benefits of this material have not been conclusive as there have been contradictory outcomes, which report both good and poor results.

Clinical studies data were influenced by variables, such as defect size, site and patient factors that were not standardised, whilst the few experimental studies carried out did not demonstrate clearly if the PRP used had comparable concentrations. A study to determine the true effectiveness of PRP in treating non- or delayed union is, therefore, necessary to justify its use in clinical practice. In this study, a standardised technique to treat a delayed union model was performed in phenotypically identical rabbits and assessed for healing using gross, histological and radiological methods to illustrate the role of PRP in enhancing bone healing.

Materials and method

Animal experiment

This experiment was conducted using 12 New Zealand white rabbits aged between 14 and 18 weeks (mean: 14.9 ± 0.8 weeks) and weighing between 2.2 and 2.7 kg (mean: 2.4 ± 0.2 kg). Approval from the animal ethics committee was obtained prior to the study, and the animals were cared for based on protocols approved by the Institutional Animal Care & Use Committee (reference number [OS/ 08/09/2008/DCSK(R)]). All rabbits were screened for common diseases, and have not been subjected to any experiments prior to this study. The experiment consisted of two stages: the first stage involved surgical procedures to create the critical-sized defect, whilst the second stage was performed to administer the different modalities of treatment.

In the first stage, similar-sized defects were created at the midshaft of the right tibias. An anterior approach was used to resect a 2-cm segment of the tibia including the surrounding periosteum proximally and distally from the bone ends. The cut was made using an oscillating saw, accompanied by saline wash to reduce osteonecrosis. Small fragment plates (2.7 mm) were used to stabilise the fractured tibia. The defects were then filled with poly(methylmethacrylate) (PMMA), which acted as a spacer to prevent bony union. The wound was irrigated using saline, and the skin closed using catgut 3/0 sutures. Postoperatively, the animals were kept separately and were given antibiotics (Kombitrim 1 ml 10 kg⁻¹). Metacham (0.3 mg kg⁻¹) was administered, according to the body weights, for analgesia. Both medications were administered postoperatively for 3 consecutive days.

In the second stage of the experiment (3 weeks following the first surgery), rabbits were divided into four groups of equal numbers (n = 3), with each group receiving a combination of treatment as summarised in Table 1.

The second surgical incision was performed at the previous surgical scar to expose the tibia defect. The PMMA spacers were removed, followed by normal saline irrigation. Treatments for each of the groups were performed, as previously described in Table 1, prior to wound closure. Kombitrim and Metacham were again administered for 3 days postoperatively.

Preparation of PRP

A total of 10 ml of blood was drawn from the central vein of the ear in rabbits from group-B and group-D and placed into ethylene diamine tetraacetic acid (EDTA) vacutainers. Centrifugation of the blood to separate the plasma from the cellular products was performed for 20 min at 150 G. The supernatant were aspirated and centrifuged for a further 10 min at 450 G. The forming bilayer of plasma containing the upper platelet-poor plasma and a lower

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**Table 1**
The summary of the groups and the treatment performed.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Control group: Second surgery was performed to remove the spacer. No bone graft or PRP was introduced into the defect</td>
</tr>
<tr>
<td>Group B</td>
<td>PRP only treated group: Upon removal of the spacer, PRP was injected into the defect site. Wound was closed without any bone graft in place</td>
</tr>
<tr>
<td>Group C</td>
<td>Bone graft only treated group: Upon removal of the spacer, bone graft was inserted into the defect. PRP was NOT injected into the defect site</td>
</tr>
<tr>
<td>Group D</td>
<td>Combined bone graft and PRP treated group: Upon removal of the spacer, bone graft impregnated with PRP in vitro was inserted into the defect site</td>
</tr>
</tbody>
</table>

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Fig. 1. A showing the centrifuged plasma which has separated into a lower layer of platelet rich plasma and a upper layer of platelet poor plasma. The region of the plasma that was aspirated is also shown. B showing the plasma that was further centrifuged to obtain the PRP and platelet poor plasma.
layer of PRP was then separated using an aspirator. Only the lower layer was used for this experiment (Fig. 1). Each vial of prepared PRP was sampled and sent to an independent diagnostic laboratory for platelet count to ensure that high concentrations of platelets were obtained in the prepared plasma. Of the six \((n = 6)\) samples obtained from the individual rabbits, it was found that the platelet numbers were between 2.62 and 4.61 (mean: 3.27 ± 1) times higher than that of the peripheral blood. PRP obtained from the rabbits were strictly administered into the corresponding rabbit from which the peripheral blood was obtained, that is, as an autologous biological product. Prior to injecting into the defect site, calcium chloride and bovine thrombin were mixed with PRP in a ratio of 1:6 for platelet activation. The calcium chloride and bovine thrombin were obtained from Tisseal® which also provided the kit (ref.: VNT1J008) for mixing the materials. Within 50–60 s, a viscous material will form within the applicator syringe and is now ready for injection into the defect site.

Preparation of artificial bone graft

The bone grafts used in this study were obtained from the National Tissue Bank in Malaysia. This artificial bone is made from cultured corals, which have been precut and sterilised to commercial standards, and marketed under the trade name Coragraft®. The choice of using Coragraft in this experiment was because of its ability to allow osteoconduction; but it lacks the osteoinduction property. This allowed the healing quality as the result of PRP to be objectively assessed without the influence of other transplanted growth factors. Prepacked Coragrafts were kept in their sterile packaging and were only exposed to the environment just before implantation during the second surgery. For group-D, Coragrafts were placed in a sterile container mixed with the activated PRP for 3 min or until a gel-like material forms enveloping the bone grafts, prior to implantation.

Gross assessment

Rabbits were euthanised at week 11 and the right tibia was harvested immediately for gross and histological examination. Union was grossly assessed using the method described by Brownlow and Simpson3 This involved a specific method of tissue handling and testing during which, the excised tibias were held horizontally and subjected to gentle digital pressure at the fracture site to determine the degree of tissue stiffness. Care was taken to not damage the excised tissues. Specimens were later stored in a deep freezer (−80 °C), whilst waiting for histology sectioning later.

Radiological assessment

Radiographic evaluations (antero-posterior and lateral plain X-ray of the tibia) were performed following the surgical procedures at week 0 (first surgery), week 3 (second surgery), week 7 (4 weeks after second surgery) and week 11 (at sacrifice). X-rays were evaluated, using a six-point scoring system developed by Cheung et al.,16 by a dedicated radiologist blinded to the study.

Histological assessments

Tissue histology was performed on longitudinal sections that included the site of the bone-defect interface. The harvested tissues were decalcified by immersing into 10% formic acid. Solutions were changed daily until complete decalcification was achieved. This process took approximately 10 days. The samples were then embedded in paraffin wax and sectioned to 5-μm thickness. Mounted histological sections were stained using haematoxylin and eosin (H&E) and Alizarin Red. The method used to stain the slides was as previously described in other literatures.14,31 Histological grading of the healed sites was based on a 14-point histological grading system described by Salkeld et al.21 The scoring was performed by an independent observer (anatomist), who is not affiliated to this study.

Analysis of results

Results were analysed using non-parametric analyses (Kruskal–Wallis and Mann–Whitney tests) due to the small sample size. A statistical package software (Statistical Package for Social Sciences (SPSS® version 17.0) was used for the analyses. Significant differences were observed when \(p\)-values were less than 0.05.

Results

No death or complications to the rabbits were observed during the entire duration of the study. Animals were allowed to ambulate within the confines of their cages until euthanasia at week 11. Upon resection of the operated tibia, no evidence of inflammation

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Fig. 2. Gross examination of rabbit tibias following euthanasia at week 11 were focused on the sites circled in these pictures. The quality of bone healing was using the grading system previously described. The alphabets within the pictures corresponds to the different groups e.g. A represents group A.
or infection was observed at the defect sites. Gross examination and grading following the described Brownlow’s system was performed. In group-A (control), there were no evidence of union and the fracture ends appeared atrophic for all three specimens (Fig. 2A). Two of the specimens showed hypertrophy of the fibula with synostosis formation. In group-B (PRP only), two tibias had stiff union and one had union. Significant amounts of callus were noted, and the fracture site appeared to have bridged. However, gaps were still present within these defect sites and were not completely filled with healed tissue (Fig. 2B). In group-C (Coragraft only), all three had stiff union. The osteotomised gap was still clearly visible despite the healing process taking place. There was also some evidence of coral resorption. Minimal callus formation was noted at the defect sites (Fig. 2C). In group-D (Coragraft and PRP), all the tibias had attained union and callus bridging within the implanted Coragraft, clearly demonstrating the superior healing of the fracture in this group as compared with the other groups (Fig. 2D).

Radiological findings are summarised in Fig. 3. Using the scoring system previously described by Cheung et al., it was observed that the mean radiological score according to weeks was highest in group-D. The Kruskal–Wallis test demonstrated a significant difference between the groups at week 4 (p-value 0.020) and week 8 (p-value 0.016). Further statistical analysis performed between the different groups at week 4 and week 8 (Mann–Whitney U) demonstrated significant difference between the groups, as summarised in Table 3. The result of the radiological grading is also summarised in Table 2. At week 7, group-B and group-D showed significantly better radiological outcome for bone regeneration compared with group-A, whilst group-D was significantly better compared with group-B and group-C. At week 11, all groups had significantly better outcome compared with group-A, whilst group-D had the best repair outcome.

Histopathology examination was performed on H&E as well as Alizarin Red-stained specimens. The control group demonstrated the least amount of bone regeneration. There was no evidence of granulation tissue, callus or evidence of early repair. There is interulsion of vasculature and distortion of marrow architecture (Fig. 4A). This is suggestive of a quiescent state within the repaired bone. In group-B, primary osteoblasts appear to line the cortex with fibrous and cartilaginous matrix (Fig. 4B). This is a good indication of osteogenic activity within the defective sites. In group-C, there was soft callus containing numerous cells resembling chondrocytes and fibroblasts. There was also the presence of inflammatory cells that suggests bone regeneration; however, there was only minimal graft–bone interaction and minimal fibrocartilaginous tissue observed within the gap spaces (Fig. 4C). The best evidence to support active osteogenesis was observed in group-D, which demonstrated a high level of osteoblast activity and formation of mineralised bone matrix; also noted was hard callus bridging within these gaps. As for the graft interaction between graft material and bone, it was observed that the histological sections performed on specimens from group-D had better bone graft interface than that of other groups (Fig. 4D).

Alizarin Red stains performed on histological sections in the control group demonstrated poor healing of the defect site (Fig. 5A). There was also no calcification or callus formation observed (Fig. 5A). In the PRP-only group, there was evidence of endochondral ossification; however, no mature bone could be seen within the defect site (Fig. 5B). Although defect callus formation was observed in specimens obtained from group-C (Fig. 5C, specimen taken from the Coragraft + PRP group (group-D) showed higher amounts of calcium deposition, suggesting an increased activity of osteogenesis (Fig. 5D). Using the scores obtained from the grading system employed in this study, statistical analysis performed demonstrated significant differences between the individual groups (Table 4). In group-D, this was significantly better compared with all other groups (p-values 0.012, 0.024 and 0.005 between group-A, group-B and group-C, respectively). It is also noted that the Coragraft group (group-C) showed the least evidence of bone union histologically, with a mean score of 4.33 ± 1.53 (this was significantly lower compared with group-B and group-D; p-values of 0.019 and 0.005, respectively).

### Discussion

Although the results are preliminary, this study demonstrates PRP’s role in treating bone defects. From both quantitative and qualitative analyses conducted using the three outcome measurements described in this study, significant differences are apparent between the PRP treated groups and all other groups. PRP is found to be effective only when used together with bone graft. This has been similarly observed in previous studies. Artifical bone graft made from cultured corals 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. Advocates feel that the benefits of using this simple-to-produce biological material will lead to better bone and wound healing with less blood loss, infection and pain. In a number of clinical trials, the use of PRP is said to enhance the rate and quality of bone defects. However, in many studies, it was found that different methodologies and techniques were used to produce PRP. In addition, there were relatively small numbers of subjects recruited in each study. This has brought concerns that the positive effects of PRPs may have been overexaggerated.

Platelets in PRP that are activated by the in vitro introduction of thrombin–calcium or the wound environment will release α-granules that contain numerous proteins that influence bone healing. These proteins include platelet-derived growth factor (PDGF-αα, ββ and αβ isomers), transforming growth factor-β

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**Table 2**

Summary of mean radiological grading at different time points. Values were based on the scoring system developed by Cheung et al. [5].

<table>
<thead>
<tr>
<th>Weeks</th>
<th>A (± 1 SD)</th>
<th>B (± 1 SD)</th>
<th>C (± 1 SD)</th>
<th>D (± 1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>7</td>
<td>1.67 ± 0.58</td>
<td>3.00</td>
<td>2.67 ± 0.58</td>
<td>4.00</td>
</tr>
<tr>
<td>11</td>
<td>2.33 ± 1.15</td>
<td>4.33 ± 0.58</td>
<td>4.00</td>
<td>6.00</td>
</tr>
</tbody>
</table>

**Table 3**

Results of Mann–Whitney U-test (p-values) comparing the radiological scores between the various groups at week 7 and 11.

<table>
<thead>
<tr>
<th>Groups (A = control, B = PRP only, C = coral only, D = coral and PRP)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.034b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.990</td>
<td>0.317</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.034b</td>
<td>0.025b</td>
<td>0.034b</td>
</tr>
<tr>
<td>Week 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.043b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.034b</td>
<td>0.317</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.034b</td>
<td>0.034b</td>
<td>0.025b</td>
</tr>
</tbody>
</table>

a Group A = control, group B = PRP only, group C = coral only, group D = coral and PRP.

b Statistically significant (p-value < 0.05).
(TGF-β, β1 and β2 isomers), platelet factor 4 (PF4), interleukin-1 (IL-1), epidermal growth factor (EGF), platelet-derived angiogenesis factor (PDAF), epithelial cell growth factor (ECGF), vascular endothelial growth factors (VEGFs), insulin-like growth factor (IGF), fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn) and many others. Secreted proteins such as PDGF provide a bioactive condition with the addition of histones and carbohydrate side chains, which then bind to the target cells, for example, osteoblasts and mesenchymal stem cells. This will lead to the activation of intracellular signalling, which directs cells to increase the cellular proliferation rate, matrix formation, collagen synthesis and cellular differentiation. The granules from platelets are released into the wound environment within 10 min of clotting, with almost all secreted within the first hour. Although the initial effects of the released proteins would last for almost an hour, the half-lives of growth factors and many other cytokines are within minutes. This will render the proteins useless unless they bind to their binding sites within that period. It is, therefore, important to ensure that the activation of platelet happens at the right time. Because platelets have a life span of between 5 and 7 days, they will continue to secrete bioactive substances at a constant rate, giving a prolonged effect as the result of cellular activation. From thereon, the effects of bone healing will be taken over by the macrophages, which are attracted by the release of PDGF. This growth factor also acts as a chemotactic agent. In addition, it has been suggested that the initial release of IGF from platelet granules results in the

Fig. 3. Radiological images of according to the different groups and time points are presented here. Note the differences in the healing ability of each group with group D demonstrating the best filling of the defect. Improvements to the bone quantity is also apparent over time.
activation of mesenchymal stem cells. These progenitor cells later transform into osteoblast-like cells that continue to provide the matrix-repair proteins. In combination with other pathways activated through similar mechanisms by platelets, healing through the use of PRP is expected to be superior. It is nevertheless wrong to conclude that the presence of platelets in wounds is a must for wound healing. In a study by Szpaderska et al., it was found that in animals that were rendered thrombocytopenic,
wound healing would continue. This is achieved by using other compensatory mechanisms, although these wounds would exhibit altered wound healing characteristics. It has also been noted that these wounds would take a longer time to heal, which may have been the observations noted in this study when comparing between group-C and group-D.

Not all studies support the finding that PRP enhances bone healing. Froum et al., Aghaloo et al. and Shanaman et al.\(^1,9,25\) all reported that they observed no differences in the healing of bone defects when PRP was used to treat conditions requiring sinus lift grafts, cranial defects in rabbits and ridge augmentations, respectively. However, Robert Marx made a very strong argument that whilst these authors have demonstrated as such, these studies either used damaged platelets, not true PRP, or have not used activated platelets or have insufficient data to make a definitive conclusion. He, therefore, adds that in these studies, the results presented may not have been a true representation of the clinical efficacy of PRP in bone healing and therefore not valid.\(^17\)

Despite the good results obtained in this study, several limitations were identified but could not be avoided. The main issue is the small number of subjects used. As a result, the study could not qualify as being an absolute indicator of proof of the effectiveness of PRP. Whilst a larger study involving more subjects would be ideal, the results obtained here does serve as a preliminary research. Therefore, this study can justify future research undertakings using larger sample size. The use of rabbits as the model may have not been the best choice when considering that these animals are quadrupeds and not bipeds. However, the use of monkeys cannot be justified and may not be necessary. Although this study have used at least three methods to measure the level of tissue healing, other modalities of measurement, which may include biochemical parameters, biomechanical testing and gene-expression analyses could provide more convincing results but at higher costs. When this study was conducted, limited financial resources was the prohibitive factor for conducting the aforementioned tests, but shall be addressed in future research undertakings when more funds are made available.

**Conclusion**

PRP enhances the repair of delayed bone unions involving critical-sized defects, but is only effective if used concurrently with bone-replacement material.

**Conflict of interest**

All the authors of the article ‘Platelet Rich Plasma (PRP) Enhances Bone Healing In Non-United Critical Sized Defects: A Preliminary Study Involving Rabbit Models.’\(^7\) are from the same institution. The sole source of the grant is from an internal fund of the university; grant number (FS125/2008A). None of the authors have any vested interest in the outcome of this paper.

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