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1. Introduction

Bone defects are a serious illness that can result after a pathological process has destroyed vital components of the bone. Most commonly the causative event is extensive trauma and subsequent infection. It can be also osteomyelitis that destroys the bone and leaves non-vital bone sequesters along the length of the bone. This damage to the bone and soft tissues heals slowly and restitution can be only expected after some time of rest and procedures of debridement.

1.1. Bone defects

Bone defect by definition is a lack of bone tissue in a body area, where bone should normally be. Lack of bone tissue results in a pseudarthrosis, artificial joint that has no physiological importance. In that area, two parts of diseased bone are joined with a fibrous tissue. That area also lacks appropriate vascularization and is usually covered with scarred or fibrotic skin [1].

Bone defects can be treated by various surgical methods. One is always constrained with fibrosis that healed a wound or the site of infection [2]. Often there are factors that impair bone healing like diabetes mellitus [3, 4], immunosuppressive therapy [5, 6], poor locomotory status and others that one has to take in account when a procedure is planned.

There are some common methods of bone defect reconstruction, like decortication, excision and fixation, cancellous bone grafting [7] and the Ilizarov intercalary bone transport method [1]. The application of these methods results in successful final outcomes as far as the bone restitution is concerned.
However, one must consider repeated surgical procedures and often long hospitalization time or frequent outpatient visits for these patients. It is also common for patients to have prolonged ambulatory impairment with suboptimal functional and aesthetic results [8, 9].

1.2. Tissue bioengineering

Tissue engineering involves the restoration of tissue structure or function through the use of living cells. The general process consists of cell isolation and proliferation, followed by a re-implantation procedure in which a scaffold material is used. Cell sources can be autologous or allogenic cells. Autologous cells are usually the better choice, because the allogenic cells could incite immune rejection by the recipient. Mesenchymal stem cells provide a good alternative to cells from mature tissue and have a number of advantages as a cell source for bone and cartilage tissue regeneration [10].

Some authors report that most tissue engineering applications in the head and neck area would probably involve the use of chondrocytes and osteoblasts along with some type of scaffold material because of the importance of initial support and shaping [11].

Theoretically, the ideal bone graft substitutes should be osteogenic, biocompatible, bioabsorbable, able to provide structural support, easy to use clinically and cost-effective. A composite graft combines an osteoconductive matrix with bioactive agents that provide osteoinductive and osteogenic properties [12].

Novel techniques have been studied recently, many involving growth enhancers with varying results. These have been used for healing wounds, ulcers, fractures, and in maxillofacial settings. Such biological enhancers are autologous platelet rich plasma (PRP) in the form of activated platelet gel and recombinant bone morphogenetic proteins (rBMP) [11, 13-23]. An animal study showed enhanced bone growth when autologous bone was combined with platelet-rich plasma [24, 25].

The healing effects of platelet rich gel were attributed to the numerous growth factors (GFs) released by the platelets after activation [19, 26]. Some of those identified are: the platelet derived growth factor (PDGF), TGF-α and β (transforming growth factor alpha and beta), EGF (epidermal growth factor), FGF (fibroblast growth factor), IGF (insulin growth factor), PDEGF (platelet derived epidermal growth factor), PDGF (platelet derived angiogenesis factor), IL-8 (interleukin-8), TNF-α (tumour necrosis factor alpha), CTGR (connective tissue growth factor), GM-CSF (granulocyte macrophage colony stimulating factor), KGF (keratinocyte growth factor), and Ang-2 (angiopoetin), as reviewed by several authors [26, 27]. The inductive potential of platelet gel in tissue regeneration could also be attributed to its significant antimicrobial activity [28].

1.3. Clinical experiments

Recent studies on patients for the regeneration of long bone and foot and ankle defects have provided promising clinical results when using platelets as a source of GFs [10]. Some studies demonstrated that with the use of platelet gel a better and stronger bone yield was achieved...
as compared to reconstruction with conventional methods [29]. X-ray images of treated bones showed increased density early in follow up and in-growth of treated area was enhanced [30].

In the majority of clinical experiments, authors have applied autologous platelets obtained by preoperative apheresis from the peripheral blood of the patient undergoing surgery. However, this may not always be the best solution. In cases of diabetes it has been shown that the release of platelet GFs is decreased in experimental diabetic animals [31]. If allogeneic platelet rich plasma was used as a source of additional GFs, healing of tissues in diabetic patients can considerably improve [32].

Allogenic single donor platelet units are easy to obtain, since they are a standard blood bank product. They are highly standardized in terms of platelet content and residual leukocyte and red blood cell content is low. All of this is due to proven centrifugal forces used for their isolation, temperature of centrifugation, techniques of separation and processing and composition of preservative solution. Also, they are available in large quantities and considered safe. Autologous platelet preparations, on the other hand, are subject to enormous variability, which hinders serious studies of their clinical efficacy [33].

We used for our procedures the standard blood bank platelet concentrates. We prepared a graft composed of allogeneic platelet gel mixed with autologous cancellous bone in order to improve the healing conditions in bone defects, which was successfully demonstrated in our pilot clinical case [34].

In our case study, we showed that the healing potential of the gel GFs obtained from a high number of allogeneic platelets could be combined with the bone forming potential of autologous osteogenic and other stem cells from the cancellous bone. We employed the plasticity of the resulting graft mixture for the modeling and all of this contributed to a successful clinical outcome.

2. Body

2.1. Problem statement

The treatment of bone defects of long bones after injury is still one of the most difficult tasks in reconstructive bone surgery. The golden standard in bone graft surgery is still the use of autologous bone graft [7]. In certain settings, especially in extensive bone defects, this method of treatment could be insufficient and could only pose an additional trauma for the patient.

Numerous authors have reported difficulties when treating defected non-unions, such as extremely long healing time and incorporation of the graft, necrosis of the grafts, and reactivation of infection [35, 36]. Concomitantly, long-term immobilization contributed to the contractures of the joints and soft tissue, and in the long-term perspective, also to the inferior functional and aesthetic results [37].

Pseudarthroses with certain mid size bone defect are complicated to treat because it is difficult to determine an appropriate treatment method. Smaller size defects can be treated with simple
bone fixation and some debridement. Larger bone defects must be treated with bone transport (Ilizarov method) or transplant of bone graft with vascular pedicle [36].

Reconstruction by vascularised bone transfer along the Ilizarov intercalary bone transport and cancellous bone grafting has been the most widely used method of treatment for large defected nonunions after injury [37, 38]. There have been several modifications of the Ilizarov method, which retain its versatility, stability and mechanics, but these methods also contribute to a high rate of complications [35, 37, 38].

Mid sized defects can be treated with cancellous bone transplant, but many limitations exist with this method. Cancellous bone is of limited availability in human body and sometimes sources have been depleted after repeated surgeries. Often, resorption of transplanted cancellous bone is seen which leads to unsuccessful bone defect bridging [39].

Bone grafts are used to replace a part of the bony defect or to enhance the healing of a fracture. Because of the inability to procure large quantities of autologous bone and the added morbidity for the patient associated with the autograft donor site, new methods of bone transplant materials have emerged in recent years [7].

Substitutes for bone defects have been tested and one of the research tasks is to devise a easily attainable promotor of ingrowth of autologous cancellous bone. Theoretically, the ideal bone graft substitutes should be osteogenic, biocompatible, bioabsorbable, able to provide structural support, easy to use clinically and cost-effective. A composite graft combines an osteoconductive matrix with bioactive agents that provide osteoinductive and osteogenic properties [39].

Synthetic substitutes that provide a scaffold to support or direct bone formation include calcium sulphate, ceramics, calcium phosphate, cements, collagen, bioactive glass and synthetic polymers. These are available in a variety of formulations, including pellets, cement and injectable paste [39, 40].

The functional properties of bone morphogenetic proteins (BMP) 2 and 7, mesenchymal stem cells (MSC), demineralised bone matrix, and biocompatible ceramics are presented in many papers describing their use in bone defect treatment [41-44]. Bone morphogenetic proteins exhibit an extraordinary power to induce new bone formation de novo without the presence of cancellous bone [45]. With their high cost, limited availability and restricted clinical indications, BMPs are a less attractive option for clinical application.

One of the clinical challenges in long bone defects is the induction of appropriate bone formation, especially in patients with diabetes. Several studies have demonstrated the clinical efficacy of various platelet derived GFs. Recent evidence shows that in diabetic patients platelets are handicapped by decreased expression of growth factors and lower potential for healing fractures [31, 46].

Although there is some evidence that the GFs are released to some extent in the stored platelet concentrates, the majority of GFs remain intact in the platelet granules if they are appropriately stored for up to 5 days [47].
The safety and efficacy of allogeneic platelets was also shown in our recent pilot case study [34]. Moreover, the preparation of autologous platelet gel requires pre-operative apheresis and blood draws from the patient, and adds to the complexity, risk and cost of surgery [48].

Based on these facts, we were of the opinion that allogeneic platelets constitute a superior alternative to autologous preparations obtained by pre-operative apheresis. Therefore, we used a standard platelet concentrate from the blood bank as a component for the activated platelet gel.

2.2. Application area

Tissue engineering involves the restoration of tissue structure or function through the use of living cells. The general process consists of cell isolation and proliferation, followed by a re-implantation procedure in which a scaffold material is used. Cell sources can be autologous or allogenic cells.

Autologous cells are usually the better choice, because the allogenic cells could incite immune rejection by the recipient. Mesenchymal stem cells are progenitor cells and can be developed in a laboratory along separate cell families. They can be differentiated into more maturated cells like osteoblasts and chondroblasts and chondrocytes. They provide a good alternative to cells from mature tissue and have a number of advantages as a cell source for bone and cartilage tissue regeneration [49].

Here we present the results of a prospective clinical study performed from May 2004 to February 2010 in the University Clinical Centre Ljubljana, Slovenia. We treated defected non-union of long bones with cancellous bone transplantation. We used allogeneic platelets as a source of additional GFs.

We treated 9 consecutive patients (3 female and 6 male), aged from 21 to 73 years (average 45.9 years), each with a defect of a different long bone (3 femoral, 4 tibial, 1 humeral and 1 ulnar). We present patients’ size of bone defect, which were classified as mid-size in a Table 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pseudarthrosis site</th>
<th>Graft volume in mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Femur</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Distal tibia</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Distal tibia</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>Femur</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Distal tibia</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>Proximal femur</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>Humerus</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>Ulna</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>Distal tibia</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Average graft volume</td>
<td>28.5</td>
</tr>
</tbody>
</table>

Table 1. Size of bone defect per patient and average of the group
Figure 1. Bone defect of distal tibia (plain X-ray)

Figure 2. Bone defect of distal tibia CT reconstruction
They had already been unsuccessfully treated with conventional methods in our or other hospitals. The therapeutic options in these cases had been exhausted. In the Figure 1 and 2, we present an example of a bone defect we treated.

In 2 of the patients, we treated osteomyelitis before applying our treatment. We took additional microbiological samples at the time of operation. Three samples were positive for pathologic bacteria and patients received appropriate antibiotic therapy. After the operation no reactivation of infection was noted. Two of the patients had diabetes on per oral therapy.

2.3. Research course

In our clinical investigation plan, the primary objective was to establish the potency of allogeneic platelet gel, from our blood bank, added to the transplanted autologous cancellous bone when treating post-traumatic mid-sized bone defect, with a follow-up of one year. The secondary objective was to investigate the healing, safety, handling and tolerance of the method and potential cost benefits.

We noted all the patients’ major variables in a protocol, radiologic examinations, and postoperative follow-up for up to one year. As a survey of the immunological side effects of allogeneic platelets, we performed a screening of HLA antibodies class I and human platelet antibodies (HPA) before the implant operation and in the third month after the operation.

2.4. Methods used

We harvested autologous cancellous bone from one or both patients’ iliac crests and ground it by hand and instruments until the particles were smaller than 5 mm. It was then stored on a sterile dish with wet gauze for later use.

For preparation of the platelet gel we used a standard allogeneic random single donor platelet concentrate that was ABO and RhD matched, serologically HIV, HBV, HCV and lues-negative, leukocyte depleted, and irradiated. A standard single donor platelet concentrate was prepared from 450 mL of whole blood, containing 70 x 10⁶ platelets in 50 mL of citrated plasma, and stored in a plastic bag designed for platelet storage at 4–8ºC on an automatic agitator for up to five days.

We performed leukocyte depletion by using a commercial filter (BioP05 Plus, Fresenius HemoCare, Bad Homburg, Germany) with 10–15% platelet loss post-filtration. We irradiated the platelet concentrate with a cobalt irradiator with 25 Gray. All platelet related procedures, including the bacteriological controls, were performed according to the recommendations for blood banking procedures.

Finally, we prepared a mixture of lightly compressed autologous cancellous bone and an equal volume of allogeneic platelet concentrate with approximately 1.4 x 10⁹ platelets per 1 mL (which is around five times higher than the physiological level of platelets in the blood).

We mixed the ingredients and added the fibrin glue components (human thrombin (100 IU/mL) in 40mM CaCl₂ (Beriplast P, ZLB Behring, Marburg, Germany)) for the activation of platelets and polymerization of fibrinogen. The implant is presented in the Figure 3. The
mixture achieved the appropriate plasticity in 20 to 30 seconds. The resulting gelatinous graft was shaped according to the defect and implanted.

Figure 3. Cancellous bone and platelet rich plasma implant

2.5. Surgical procedure

In all our operations, we approached the bone defects through previous surgical incisions after administering a single dose of prophylactic antibiotic. After debridement of the non-union which is presented in Figure 4, we filled the resulting bone defect with a semi-solid, moldable gelatinous graft, presented in the Figure 5.

Figure 4. Bone defect at the operation
We revised the method of fracture fixation and repositioned bone fragments were and fixed them in good alignment. We applied a different fixation method where it was necessary or inadequate and we present the fixation methods in Table 2.

Table 2. Fixation methods used and graft volume per patient

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pseudarthrosis site</th>
<th>Fixation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Femur</td>
<td>Internal plate</td>
</tr>
<tr>
<td>2</td>
<td>Distal tibia</td>
<td>External fixator</td>
</tr>
<tr>
<td>3</td>
<td>Distal tibia</td>
<td>Internal plate</td>
</tr>
<tr>
<td>4</td>
<td>Femur</td>
<td>Tutor brace</td>
</tr>
<tr>
<td>5</td>
<td>Distal tibia</td>
<td>Internal plate</td>
</tr>
<tr>
<td>6</td>
<td>Proximal femur</td>
<td>Dynamic hip screw with long plate</td>
</tr>
<tr>
<td>7</td>
<td>Humerus</td>
<td>Internal plate</td>
</tr>
<tr>
<td>8</td>
<td>Ulna</td>
<td>External fixator</td>
</tr>
<tr>
<td>9</td>
<td>Distal tibia</td>
<td>Internal plate</td>
</tr>
</tbody>
</table>

We placed negative pressure suction subcutaneously, away from the graft in order to minimize the removal of GFs. All procedures were carried out within a sterile operation field - aseptic conditions.

In the follow-up protocol, we assessed the general status after the operation, and the bone configuration with X-ray at 2, 4, 6, and 12 months. We assessed bone remodeling at 6 and 12 months by CT scan. We drew blood samples from each patient at week 14 for the identification of anti-HLA/Class I antibodies and anti-HPA antibodies in order to assess potential immune reactions related to the use of allogeneic platelets. We used the standard in-house platelet immuno-fluorescence test (PIFT) and antigen capture ELISA test (PAK-12, GTI, Brookfield, USA) to screen for antibodies.
2.6. Results

We removed the drains on the second or third day after the operation, draining different volumes, on average 250 mL (200 to 450 mL). Immediate post-operative care was uneventful in all cases. We discharged the patients 6-8 days after the operation and they were regularly examined in the outpatients’ clinic.

Out of 9 patients, 7 successfully healed their defect with the implant (78%). Figure 6 shows healed bone defect in distal tibia.

![Healed bone defect after grafting and platelet additive](image)

Different healing times are presented in Table 3.

<table>
<thead>
<tr>
<th>Time in weeks</th>
<th>Minimal</th>
<th>Maximal</th>
<th>Average</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to appearance of hazy callus</td>
<td>6</td>
<td>24</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Time of partial weight bearing</td>
<td>12</td>
<td>40</td>
<td>22.5</td>
<td>18</td>
</tr>
<tr>
<td>Time of free mobility and full weight bearing</td>
<td>16</td>
<td>48</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Time of overall bone healing</td>
<td>16</td>
<td>36</td>
<td>23</td>
<td>24</td>
</tr>
</tbody>
</table>

*Table 3. Healing times after the operation for successful cases (time in weeks)*

We noted major complications during the treatment in 3 patients (33%): poor incorporation of implant, mental deterioration leading to non-compliance, and radial nerve palsy, which receded (1 patient respectively). Two of these patients had to undergo further surgery. More detailed data of bone healing are displayed in Table 4.
Table 4. Detailed bone healing data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time to appearance of hazy callus (weeks)</th>
<th>Time to partial weight bearing in defects (weeks)</th>
<th>Time to free mobility and full weight bearing (weeks)</th>
<th>Extent of graft incorporation</th>
<th>Time to stable bone healing (weeks)</th>
<th>Complications during treatment</th>
<th>Major complication</th>
<th>Final result (12 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F/63</td>
<td>8</td>
<td>12</td>
<td>40</td>
<td>complete</td>
<td>16</td>
<td>none</td>
<td>no</td>
<td>healed</td>
</tr>
<tr>
<td>2/M/50</td>
<td>8</td>
<td>18</td>
<td>32</td>
<td>proximally 1/4, distally complete</td>
<td>24</td>
<td>left hip fracture</td>
<td>no</td>
<td>healed</td>
</tr>
<tr>
<td>3/F/49</td>
<td>10</td>
<td>16</td>
<td>-</td>
<td>complete proximally, distally not at all</td>
<td>24</td>
<td>pseudarthrosis, re-operated</td>
<td>yes</td>
<td>failed</td>
</tr>
<tr>
<td>4/M/45</td>
<td>24</td>
<td>32</td>
<td>44</td>
<td>both ends 1/2</td>
<td>28</td>
<td>none</td>
<td>no</td>
<td>healed</td>
</tr>
<tr>
<td>5/F/45</td>
<td>8</td>
<td>40</td>
<td>48</td>
<td>proximally ½, distally not at all</td>
<td>36</td>
<td>poor compliance, mental disorder, pseudarthrosis, re-operated</td>
<td>yes</td>
<td>failed</td>
</tr>
<tr>
<td>6/M/73</td>
<td>6</td>
<td>16</td>
<td>24</td>
<td>complete</td>
<td>24</td>
<td>extremity shortening</td>
<td>no</td>
<td>healed</td>
</tr>
<tr>
<td>7/M/33</td>
<td>8</td>
<td>-</td>
<td>16</td>
<td>complete</td>
<td>16</td>
<td>n. radialis paresis</td>
<td>yes</td>
<td>healed</td>
</tr>
<tr>
<td>8/M/21</td>
<td>12</td>
<td>-</td>
<td>16</td>
<td>complete</td>
<td>16</td>
<td>none</td>
<td>no</td>
<td>healed</td>
</tr>
<tr>
<td>9/M/34</td>
<td>8</td>
<td>18</td>
<td>30</td>
<td>complete</td>
<td>24</td>
<td>none</td>
<td>no</td>
<td>healed</td>
</tr>
</tbody>
</table>

No side effects caused by the implant were observed; no platelet or HLA-class I antibodies were detected in any patient on follow-up.

We observed the survival of the implant to be excellent; most of the volume of the implant was preserved. Of clinical importance are ingrowths of the implant into adjacent bone. This was critical in the case of distal tibia (patient 2, 3, 5, and 9), where we observed diminished incorporation in the distal part, where the metaphysis of the tibia is less vascularized. In the case of femur pseudarthrosis (patient 6), bone quality was insufficient for the implant to regenerate the whole bone circumference, so healing was prolonged.

In theory, allogeneic platelets could have several certain side effects. In order to minimize these, all platelet units in our study were leuko-depleted and irradiated in order to prevent immune and bacteriological side effects, especially alloimmunisation to HLA-Class I and HPA antigens [50]. In fact, there was no evidence of immune reactions or transfusion-transmitted infections following the procedures. There have been no signs of bacterial contamination, which is not strange, based on the recent observation that the platelet gel exhibits significant antimicrobial activity in vitro [28].

The combined autologous/allogeneic graft showed successful incorporation into the defective pseudarthrosis in 7 out of 9 patients, which was confirmed with the CT scans and plain X-ray
film. The problem with the two patients in whom the therapy failed was the poor incorporation of the graft in the distal tibia, where bone healing is compromised through many factors [51].

One of the patients had a deteriorating psychiatric disorder and could not follow instructions later in the study, and one had a poor bone situation arising from previous treatments. The other seven patients with successful outcomes achieved a satisfactory clinical improvement with no side effects related to the procedure.

A bacterial infection did not reoccur in cases where an infection was previously treated. Our treatment has concluded prolonged ongoing hospitalizations and immobilizations for some patients who previously underwent numerous operations and rehabilitations. Only one patient had to be reoperated only once again, because of poor implant ingrowth.

2.7. Further research

As bioengineering techniques improve and become more clinically applicable, so does the field of application expand. In our work we have shown one of the methods to be useful in treating mid-size bone defects.

Further application of platelet rich plasma as a source of growth factors can be used in other settings where tissue defects exist. It is a natural derivative like blood transfusion and can be applied on the part of the body, where natural mechanisms would need some bioengineering support.

Further investigation should be directed into measuring the comparative efficiency of this treatment. It should be compared to golden standard treatment and determine also novel applications in bigger and smaller defects.

3. Conclusion

We showed that adding a platelet gel to a cancellous bone graft can help in retaining grafted bone from resorption and enhances its incorporation into adjacent bone. The standard platelet concentrates from the blood bank did not pose a significant risk for the affected patient. The results indicate good reasons for the application of this method in the treatment of bone defects in long bones.

This is the first report of a prospective clinical study monitoring the use of allogeneic platelets mixed with autologous cancellous bone for the treatment of the non-union of long bones after fractures. Our new method of tissue engineering seems to have the potential to become a widely approved and accepted method of bone tissue replacement in the treatment of the non-union of long bones.

Last, but not least, it is worth noticing that the outdate rate of the platelet units is currently in the range of 8-27% of all prepared platelet units [52] This leads to the conclusion that the successful use of allogeneic platelets would significantly decrease the amount of wasted platelets, which could consequently favorably change the results of blood banking policies.
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