



The Synergistic Osteogenic Potential of Platelet Rich Plasma (PRP) and Bone Chips Autograft Promotes Bone Regeneration *In vivo*: A Complex Large Cranial Defect Study in Child and Review of the Literature

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Abstract

Large calvarial bone defects represent a significant challenge in children. Often complicated by a shortage of bone and a contraindication to the use of synthetic materials. All strategies show limitations. This study proposes an effective method for large/complicated/stable cranial defects based on the synergistic combination of Platelet Rich Plasma (PRP) with autogenous bone chips. PRP has been used in alveolar bone regeneration and in orthopedic surgery. Autogenous bone grafts has been used in specific bone reconstruction (e.g. plastic surgery). We hypothesized that addition of growth factors would enhance the *in vivo* effects of bone grafts in promoting skeletal tissue repair. We compared the *in vivo* osteogenic potential of iliac bone particulate compared with calvarian bone shavings (generated by using bone scraper) both associated to PRP. The case study is the skull regeneration in an 11-years-old girl with complex and severe established craniolacunias after bone destruction caused by Candida, in which PRP with particulate bone grafts reconstructed the anterior defect, and PRP with shavings bone grafts reconstructed the posterior defect. Big cranial defects were filled and minimal unfilled defects served as controls. After 4 weeks, only those defects filled showed significantly increased bone regeneration, compared to unfilled control defects, as judged using radiology and histology. CT images showed the remarkable potencies of PRP used in conjunction with bone autograft, to fill a large calvarial defect and to stimulate osteogenesis. Successful bone regeneration was achieved with an expansive bone formation, which went on six months after the procedure, and evident bridges of osteointegration, in both anterior and posterior defects. Histology examination by biopsy demonstrated the presence of vascularized cortical and spongy bone. These data suggest that assembling PRP and autogenous bone is a suitable cellular scaffold for regeneration of cranial bone tissue in a one surgical step, in a one therapeutic approach. The study showed an effective cranioplasty for stable large cranial defects both with particulate and with shavings. This evidence has a positive effect on the clinical practice because the bone scraper allows an easy and safe harvesting of grafts avoiding the creation of minor resistance areas and obtaining a large volume of bone substance.

Keywords: Skull reconstruction; Bone regeneration; Calvarial/cranial defects; Platelet rich plasma; Bone graft; Bone scraper

Introduction

Large calvarial defects represent a significant challenge to craniofacial surgeons. Especially difficult to manage are defects in children where the reconstruction is frequently complicated by a shortage of bone. This problem is most apparent after approximately 2 years of age, when the dura loses/diminishes its osteogenic potential and the diploic space has not matured to the point to support the harvest of criterion standard split-thickness calvarial grafts [1].

In humans, despite existence of a variety of natural and synthetic biomaterials, and the development of techniques for bone tissue engineering, all reconstructive strategies show restrictions [2-4]. In animal (rabbit and rat models), relative successful repair of large-scale

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calvarial defects, have been reported with the use of stem cells and bone morphogenetic protein, and the use of bisphosphonates like zoledronic acid to control the bone resorption [1,5-7]. Recently, Saha et al., [1] showed that a biomimetic self-assembling peptide promotes bone regeneration *in vivo* in rat cranial defect study. However, bone tissue engineering practices have not proceeded to clinical practice due to several limitations or challenges. Specifically, the research groups discuss widely about biomaterial scaffolds, micro- and nano-structural properties of the scaffolds, the incorporation of biomimetic properties and/or growth factors, the cellular approach including the use of mesenchymal stem cells, embryonic stem cells, adult stem cells or induced pluripotent stem cells. A main challenge that faces the bone tissue engineering field is the lack of sufficient vascularization at the defect site [6]. The bioengineering has seen an emergence of literature nevertheless the viable products appear distant from routine scenario. Today, several authors use implants (e.g. custom-made titanium implants, porous hydroxyapatite, and other alloplastic materials) because they have advantages, such as ready availability and no donor-site morbidity, but are associated with higher infection and extrusion rates than are autogenous materials [9,10]. Other drawbacks can be dislocation and visibility of the implant from the skin. Moreover, their use is limited in children by their inability to grow with the developing craniofacial skeleton.

Autogenous bone graft is the gold standard method of tissue replacement in bone defects. It uses all three known mechanisms of bone regeneration (osteogenesis, osteoinduction, and osteoconduction). (Khan) It is non-immunogenic and its superiority comes from the transfer of osteocompetent cells [11,12]. Exchange cranioplasty using autologous calvarial particulate bone graft appears effective to repair complicated large cranial defects (>5 cm²) but it creates new areas of minor resistance in the skull [13]. We propose a different effective method for large/complicated/stable cranial defects that we propose for difficult defects in children. Currently, our approach to repair bone defects in clinical practice relies upon the combination of Platelet Rich Plasma (PRP) with autogenous bone grafts. In this article, we report the case of skull regeneration in an 11-years-old girl with severe craniolacunia after bone destruction caused by Candida. In this case of large and stable cranial defects, we compare the cranial bone regeneration by using PRP with particulate versus shavings bone grafts. We would like to report our clinical experience because there is no comparative study of the *in vivo* osteogenic potential of iliac bone chips compared with calvarian bone shavings (generated by using bone scraper) both associated to PRP. This study followed the Declaration of Helsinki and it was approved by the Bambino Gesù Children's Hospital.

Complex large cranial defect study

At 10 months old, a newborn girl with Apert syndrome (Figure 1A) underwent facial bipartition osteotomies with reshaping to symmetrize the orbital region (Figure 1B). At 5 years old, she underwent anterior cranial vault, monobloc osteotomies with advancement and orthognathic surgery. Kamamoto midface distractors were utilized for progressive advancement of the maxilla. One month later, she had fever persisted and an increase in the serum alkaline phosphatase level. The medical examination highlighted maxillary microabscesses with production of secretions. Biopsy specimens of the secretions identified the causative agent as *Candida albicans*. Therefore, she underwent removal of infected distractors and bone necrosis areas of the maxilla and the skull. She was treated with fluconazole 6 mg/kg/die intravenous, and after 15 days of

therapy, her body temperature and serum alkaline phosphatase level were normal. At home, she continued the same dosage per os. At 12 months follow-up, skull CT scan showed severe craniolacunia after devastating bone destruction caused by *Candida*. This condition was stable with no trend to improve in the following 5 years in which parents refused interventions (Figure 1C). At 11 years old, the patient underwent reconstruction of the established large calvarial defects, using combination of Platelet Rich Plasma (PRP) with autogenous cortical and cancellous bone chips.

Bone chips harvesting

Sources of bone grafts were iliac and calvarial bones. Cortical and cancellous grafts were harvested from anterior iliac crest bilaterally and transformed into particulate. Cortical bone chips were directly harvested from outer cortical skull such as shavings by using a bone scraper, near bone defects also. PRP with particulate bone grafts reconstructed the anterior defect, and PRP with shavings bone reconstructed the posterior defect.

PRP preparation

The thrombocyte solution used in these cases has traditionally been supplied by the blood bank. Vivostat is a system for preparation of autologous fibrin sealant or platelet rich fibrin from 120 ml whole blood. Furthermore is possible use it for preparation of those omologous products where the patient's blood isn't good in term of hemoglobin or platelets count. The automated system prepares 6 ml of product, platelet rich fibrin or fibrin sealant. For omologous product, is possible prepare it before the use and store at -30°C. In our case report, we used omologous platelet rich fibrin because was necessary two sample (12 ml) of product. It was prepared from donor's fresh frozen plasma and platelets units. After preparation the two simples was stored at -30°C and thawed on surgical day. The choice of platelet rich fibrin was: 1) platelet rich fibrin has platelet growth factors 2) platelet rich fibrin is strong sealant. After thawed of platelet rich fibrin, it was sprayed on cranial bone with bony scales.

Postoperative computed tomographic scan

A computed tomographic scan with three-dimensional reconstructions was planned at 1-month (Figure 2A) and 6-months (Figure 2B) follow-up postoperatively. CT images demonstrate the remarkable potencies of PRP used in conjunction with autograft of bone chips, to fill a large calvarial defect and to stimulate osteogenesis. Successful bone regeneration was achieved with an expansive bone formation, which went on six months after the bone graft, and evident bridges of osteointegration (Figure 2B). Significant bone growth was identified on the serial scans, with significant osseous coverage of the large skull defect.

Histology

Biopsies were performed at 6-months follow-up. Histology examination demonstrates vascularized cortical and spongy bone in the biopsy, remodeling activities and new bone formation by apposition and live osteocytes (Figure 3A-3C), in both anterior and posterior defects. The morphological picture corresponds to the "creeping substitution" process. The preexisting dead bone, recognized by empty lacunae devoid of osteocyte nuclei acts as a scaffold for deposition of new living bone with osteocytes in the lacunae. Prominent arcuated cement lines of arrest type are evident between viable and necrotic bone. Bony trabecular is rimmed with active osteoblasts, while on the contrary only few osteoclasts are visible.

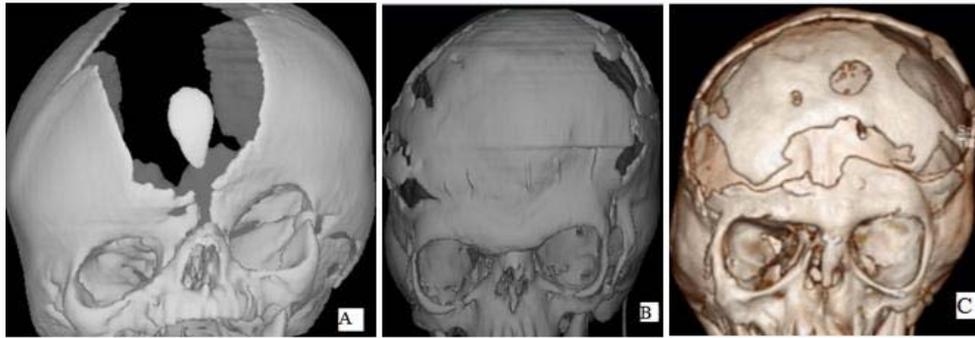


Figure 1: CT scan views of the patient with Apert syndrome (A, preop) underwent facial bipartition osteotomies with reshaping to symmetrize the orbital region (B, 3-years postop) and (C) CT scan views at 11 years of age of the skull with bone destruction by Candida.

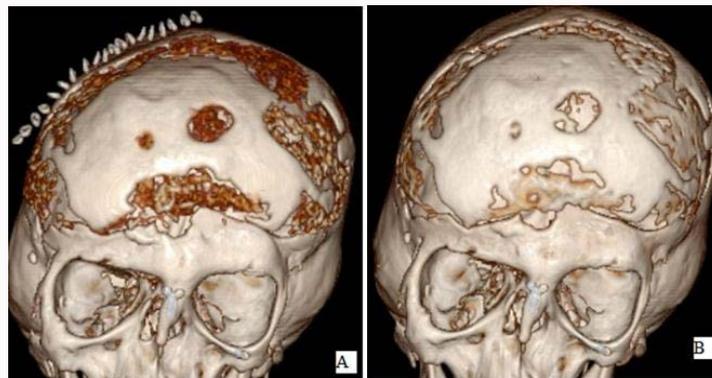


Figure 2: Postoperative CT scan: immediate post-operative (A, bone grafts in place combined with PRP) and 6-months follow-up (B, bone regeneration and bridges of osteointegration).

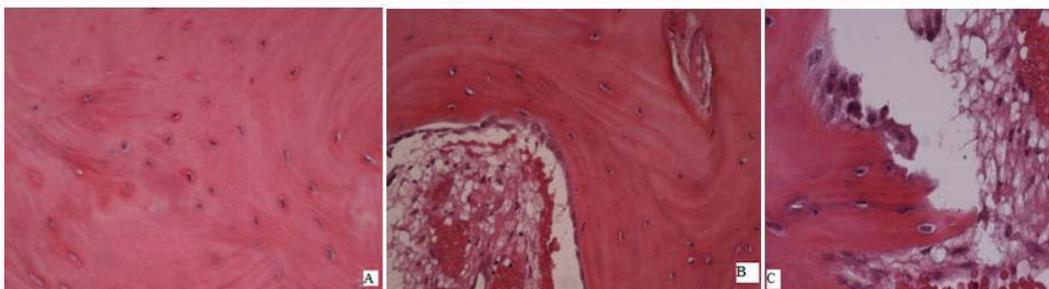


Figure 3: Histology: A) Viable and dead bone separated by curved cement lines of arrest type; B) Osteoblasts with appositional activity are present along the surfaces of trabeculae; C) Osteoclastic resorption combined with deposition of new bone matrix by osteoblasts.

Discussion

Fungal infections are emerging as a growing threat to human health, especially in immunocompromised patients. Candidiasis is the most common fungal disease among hospitalized patients in the developed world [14-16]. Deep-seated candidiasis arises from either hematogenous dissemination or direct inoculation of candida species to a sterile site, such as the skull. The invasion of the skull evolves towards devastating bone destruction. The medical treatment should be early and prolonged, because the destruction can be massive. The surgical management of a large calvarial defect represents a challenge, especially in children. Closure of calvarial defects in children is problematic because the supply of autologous bone is limited and alloplastic materials are not advocated. Several authors propose alloplastic materials, but despite advancements in materials and techniques used for cranial reconstruction,

complication rates following cranioplasty remain significant [17,18]. Alloplastic materials are available but are limited by their inability to grow with the developing craniofacial skeleton and by their susceptibility to infection and extrusion. This clinical problem would benefit significantly from the development of an off-the-shelf tissue replacement solution, nevertheless none of the approaches proposed thus far have proved effective in humans large bone defects. The proof of concept for repair of critically sized bone defects using tissue-engineered bone graft substitutes has been provided by a number of animal studies, and several clinical studies have been conducted to assess the safety and efficacy of this approach in man [19-23]. Nevertheless, bone-tissue engineering did not find its way to routine clinical practice [24]. Therefore, surgeons must everyday study as improvement the existing techniques, until to the complete development of the techniques of bone tissue engineering and gene therapy that have indicated great promise for the bone regeneration

purpose [2,25]. The osteogenic potential of bone dusts is lower than that of bone chips, and the absorption speed of bone dusts *in vivo* is faster than that of iliac bone chips. The increased resorption speed appeared to result from an increase in osteoclast cell number. Therefore, caution needs to be used when surgeons employ bone dust as a bone graft substitute [26]. Currently, the gold standard of therapeutic strategies is still autografts. Several authors consider the pediatric calvaria reconstruction complicated by a shortage of autologous bone. Greene previously showed that cranial particulate bone graft applied over the coronal gap during primary fronto-orbital advancement reduced the incidence of residual osseous defects [12]. In case of large (>5 cm²) or complicated cranial defects, these authors propose an autologous exchange cranioplasty technique using full-thickness calvarial bone from the intact cranium to repair the original defect, and particulate bone graft to cover the donor site [13]. This technique is effective for reconstructing large cranial defects but inevitably creates an additional area of least resistance in the skull.

An alternative is the association between PRP and bone grafts. Especially interesting are: 1) The bone chips/shavings harvested by scraper from intact cranium that increase much the grafts available without creating areas of less resistance in the skull. The harvest is easy without specific complications and difficult. 2) Bone chips/shavings may or may not be associated with bone particulate, when there is PRP. This study shows the equivalent osteogenic potential of shavings and particulate bone graft, when both are associated with PRP. 3) Platelets and growth factors have been used for many years to help regenerate tissue and stimulate bone formation in e.g. orthopedic surgery [27]. Platelets release many substances that promote the tissue repair and the formation of new blood vessels. It's known that platelets play a fundamental role in the healing mechanisms thanks to the ability to release growth factors, including PDGF, TGF β , VEGF, IGF-1, FGF, and EGF. Moreover, the granules contained in the platelets are a source of cytokines, chemokines and many other proteins variously involved in stimulating the cell proliferation and maturation, in modulating the inflammation, and in regulating the tissue homeostasis and regenerative processes. We believe the PRP realizes optimum microclimatic conditions for graft viability and its growth factors can stimulate or trigger the bone regeneration. So, we consider PRP as an optimal scaffold vehicle for engraftment, delivering osteogenic cells, osteoconductive materials and osteoinductive growth factors.

Osteoinduction first received serious attention when Urist demonstrated that Bone Morphogenetic Proteins (BMP's) in decalcified bone matrix were able to induce bone formation in muscles of rodents [28]. Soon afterward, BMP's were identified as the bone-inducing factors [29]. Later it was documented that heterotopically implanted synthetic and xenogenic Calcium Phosphates (CaP) biomaterials may also lead to bone formation [30-34]. The exact mechanism through which CaP biomaterials without osteoinductive proteins induce bone formation is not clear. And the amount of heterotopic bone formation observed on CaP biomaterials is significantly less than the amount that can be induced by BMP's [35]. In addition, there has been discussion of whether the apatite crystals observed on the CaP surface represent true osteo induction or are the result of a seeding phenomenon, where circulating osteoblast precursors are attracted to the osteophilic CaP surface [36,37]. Bone morphogenetic proteins-based tissue engineering is a viable approach to craniofacial reconstruction. Smith et al. evaluate and compare the relative efficacy of adipose-derived stem cells, bone morphogenetic

protein (BMP)-2, and adipose-derived stem cells osteo induced with BMP-2 in the context of repairing large-scale calvarial defects in the pediatric population (square, 15 mm per side, rabbit calvarial defects). Although theoretically promising for craniofacial applications, adipose-derived stem cells have not yet proven practically useful in this arena. It is possible that adipose-derived stem cell-based therapies may eventually be developed to capitalize on the unique characteristics of the calvarial milieu in reference to osseous healing potential. Although they have met with success in using BMP-2 for large-scale calvarial reconstruction, these reconstructions require enormous doses of BMP and are not refined enough to allow for detail-oriented repair of intricate craniofacial structures [1]. While hASCs can be utilized to heal an acute mouse calvarial defect, hASCs do not enhance healing of an established (or chronic) defect. Endogenous BMP signaling activated post-injury may explain these differences in healing. Platelet rich plasma enhances osteogenic differentiation of hASCs *in vitro* and may prove a promising therapy for future skeletal tissue engineering efforts [38]. Concerning the particulate from bone scrapers, the concentration of growth factors known to be involved in bone formation, like BMP-2 and VEGF, has been demonstrated to be higher in autografts prepared by bone mill and bone scraper than with piezosurgery and bone filters [39]. Calvarial bone is a readily available source of bone, though the calvaria consist mostly of cortical bone. A bone scraper can be used to create a copious amount of "cancellous"-like bone, when it is combined to PRP [40]. Platelet-Rich Plasma (PRP) has a pool of multiple growth factors efficient at inducing the proliferation and osteogenic differentiation of human adipose-derived stem cells (hADSCs). Bone morphogenetic protein (BMP)-2 is a strong stimulator for the osteogenic differentiation of hADSCs [27]. It is known that platelets are a source of several growth factors. This fact stimulated the development of a platelet concentrate with the intention of increasing the levels of local growth factors delivery, which, theoretically, if present at a damaged site, could improve the healing process.

Several authors started to use the Platelet-Rich Plasma (PRP), mainly in association with autogenous bone graftings in orthopedics, and also to improve soft tissue repair [27,41]. Although there is still no consensus about the ideal platelet concentration that could optimize the tissue repair process, some *in vivo* and *in vitro* studies suggest that a PRP highly concentrated could even be harmful to the repair. In PRP experimentation there is a concern about the employed method of concentration regarding the ability to really concentrate platelets. It is also important not to damage the platelets during the process. Thus, more studies *in vivo* and *in vitro* may contribute to clarify aspects of PRP use and its effectiveness [42,43]. Our results suggest that the association between bone shavings and PRP is an effective combination and a viable approach to craniofacial reconstruction and it can improvement in our ability to repair large bone defects.

Conclusion

The authors report the case of skull regeneration in an 11-years-old girl with severe craniofacial defects. The synergistic osteogenic potential of PRP and autograft of bone chips allows significant osseous healing. This effective combination is a viable approach to craniofacial reconstruction. The advantage of our study is to show the use of bone scraper and PRP guarantee an autologous reconstruction in condition of apparent shortage and large defects.

This method is effectiveness alone, but it can be used also for enhancement of the autogenous particulate bone graft for inlay

cranioplasty. This pilot study provided encouraging results; so, we will performed a study based on a larger sample and would like this article could be the starting point for multicenter studies, in order to validate the surgical technique.

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