Growth Factors Release From Concentrated Growth Factors: Effect of β-Tricalcium Phosphate Addition

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Background: Platelet concentrates represent a new approach to improve tissue regeneration and can be used alone or together with autogenous bone, recombinant human growth factors, and/or other biomaterials, to enhance tissue regeneration. Among platelet concentrates, concentrated growth factors (CGFs) exhibit an interesting clinical and biotechnological application potential.

Objective: The aim of this study was to evaluate the in vitro release of 4 growth factors (bone morphogenetic proteins [BMP] -2, BMP-7, transforming growth factor [TGF] -β1, and insulin-like growth factor [IGF] -1) by the enzyme-linked immunosorbent assay (ELISA) technique, in CGFs mixed or not with β-tricalcium phosphate (β-TCP), using or not the Round-up device, at different times.

Methods: CGFs were obtained from healthy volunteers, mixed or not with β-TCP, using or not the Round-up device. The release of 4 growth factors from these CGFs was then measured at 5 hours, 1, 3, 6, and 8 days, using the ELISA assay.

Results: Comparison of the results obtained with those achieved for CGFs alone showed that BMP2 and BMP-7 release, significantly increased in CGFs mixed with Round-up and β-TCP, TGF-β1 release was similar to CGFs alone, whereas IG-1 release was lower compared with CGFs alone.

Conclusion: The present data suggest that β-TCP addition to CGF could enhance and improve tissue regeneration, especially bone regeneration, increasing the release of some growth factors that play an important role in osteogenesis.

Key Words: β-tricalcium phosphate, CGF, growth factors release, round-up

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The successful placement of dental implants revolves around the availability of adequate bone at the time of implantation. The majority of dentoalveolar bone loss occurs in the 6 months following tooth extraction, accounting for loss of approximately 40% of alveolar height and 60% width.[1] This inadequate bone is associated with decreased treatment success rates leading to increased research into materials and techniques to maximize the available bone and regenerate the lost bone.[2] The biological principles that can potentially contribute toward this regeneration are: osteogenesis, osteoinduction, and osteoconduction.[3,4] Osteogenesis is the process in which new bone is synthesized by cells of the graft (mostly surface cells surviving transplantation) or from cells of host bed.[3] In osteoinduction, there is induction of the host bed resulting in migration and proliferation of undifferentiated mesenchymal cells in the site to be regenerated. This process is driven by the growth factors and other proteins derived from the host. The osteoconduction allows the provision of a 3-dimensional scaffold to guide the tissue regeneration via ingrowth of capillaries, perivascular bed and osteoprogenitor cells from the host.[5] Autologous bone grafting is considered as the ''criterion standard,'' which allows all 3 of the above biological principles for bone regeneration to take place.[6] However, the postoperative morbidity and complications associated with donor site along with substantial cost limit its use.[6,7] Allogenic bone grafts are alternative, but they have limited osteoinductive properties and there are issues of immunogenicity and rejection reactions.[6] Owing to the above limitations, significant research has been conducted to investigate alternative strategies and materials to augment the bone-regeneration process. One of the extensively studied area is the clinical application of bioceramics in bone grafting and tissue engineering since the revolutionary work was conducted during 1970s and 1980s. These materials have osteoconductive potential, thus providing a scaffold for bone repair and bone regeneration.[8] Common bioceramics are: glasses, glass ceramics, and calcium phosphates.[9,10] Literature suggests that calcium phosphate bioceramics including hydroxyapatite (HA) and tricalcium phosphate (TCP) are unique, as they induce a biological response similar to the one generated during bone remodeling and result in superior stimulation of bone formation and bone bonding.[11,12] The use of beta TCP (β-TCP) as a bone substitute has been growing in recent years.[13–16] It is an alloplastic material widely used in periapical surgery to enhance new bone formation, demonstrating superior biocompatibility and osteoconductivity in both animal and clinical studies.[17,18] A number of studies have also combined the bioceramics with growth factors, other proteins, and cells to promote tissue regeneration and bone healing.[19–21] This combination has been shown to significantly enhance new bone formation because of 3-dimensional stability of the graft coupled with the release of the growth factors at the site. These growth factors secreted by platelets play a crucial role in cell migration, cell proliferation, and angiogenesis for tissue

regeneration and thus wound healing and bone formation.[22–27] Among them, the superfamily of β-transforming growth factors (TGF-β) is especially related to bone regeneration, which includes transforming growth factors β1, β2, β3 (TGF-β1, β2, and β3) and the bone morphogenetic proteins (BMPs).[28] BMPs have been especially investigated due to their osteoinductive nature.[29,30] Another growth factor, insulin-like growth factor-1 (IGF-1) has been shown to play an important role during fracture healing. In a study of Wang et al,[31] IGF-1 signalling during fracture healing was investigated in an osteoblast-specific IGF-1 receptor (IGF-1R) conditional knockout (KO) mouse model, showing that IGF-1R is not only involved in osteoblast differentiation during fracture repair, but it also plays an important role in coordinating chondrocyte, osteoclast, and endothelial responses that all contribute to the endochondral bone formation required for normal fracture repair. Previous research has used various preparations of growth factors including platelet concentrates such as platelet-rich plasma (PRP), platelet-rich fibrin, and concentrated growth factor (CGF) for reconstruction of bony defects.[32–36] These platelet concentrates are blood derivatives,[37,38] prepared from the patient's own blood which not only enhance tissue healing, but also improve the clinical outcomes of various surgical procedures, reducing complications such as pain, inflammation, and morbidity.[39,40] Sohn et al[41] demonstrated that the use of CGF without any other grafting material in sinus augmentation resulted in fast new bone formation that was histologically dense and mature owing to their greater tensile strength, higher viscosity, and superior adhesive strength.

Similar results were shown by Mirković et al[33] who concluded that CGF can be used alone or mixed with a bone graft for reconstruction of bone defects. They also emphasised that the method is fairly simple, without risk of transmissible and allergic diseases and cost-effective. In another work,[42] PRP with calcium phosphate granules showed to promote metaphyseal bone healing in mini-pigs. However, the ability of the bioceramic scaffold to deliver the growth factors relies on the release and controlled administration of these growth factors from the scaffold.[43] An animal study was carried out by Suárez-González et al[44] to investigate the controlled release of vascular endothelial growth factor and BMP-2 from β-TCP scaffolds and it was found that increase in the blood vessel ingrowth occurs in a dose-dependent manner. Another study on the adsorption behavior and release characteristics of growth factors from different biodegradable implants suggested that the release kinetics of the growth factors from the biodegradable implants is a 2-stage process.[45] There is very rapid release during the first hours, which is followed by a specific release depending on chemical/physical interaction of the material and the growth factor used.

Based on these scientific evidences and considerations, the aim of the present study was to investigate the in vitro cumulative release of 4 growth factors (BMP-2, BMP-7, TGF-β1, and IGF-1) from CGFs after mixing with β-TCP, using a specific Round up device (Silfradent srl, Forlì, Italy).

MATERIALS AND METHODS

The research was carried out by collaboration between BPP University and the Department of Clinical and Experimental Sciences of the University of Brescia. The experiments were carried out at the Laboratory of Anatomy and Physiopathology (University of Brescia), from May 2015 to October 2015. The research was conducted according to the principles of the Declaration of Helsinki.

Blood Collection

For the experiments, the venous blood was collected from antecubital fossa region of 2 healthy volunteers (V1 and V2) following standard sterile techniques and using a 21-gauge needle. Both volunteers were healthy adult males (age range between 24 and 29 years) of white ethnicity, with platelets, red blood cells, and leukocytes levels within the normal range and who have expressed their informed consent. The volunteers were screened for the exclusion criteria which were: systemic disorders, smoking, infections, nonsteroidal anti-inflammatory drug use, hemoglobin level <13.5 g/dL. The collected samples were always processed immediately after blood sampling.

CGF Preparation

The CGFs were produced as follows: 9 mL of blood was drawn into each sterile Vacuette tube (Greiner Bio-One, GmbH, Kremsmunster, Austria) coated with microscopic silica particles to activate clotting. 10 tubes were obtained from each participant and were then immediately centrifuged in Medifuge (Silfradent srl, Forlì, Italy), which has exclusive characteristics including those related to acceleration/deceleration, speed, angle of test tubes, and working temperature.[37,46] It uses the programme with the following characteristics: 30-second acceleration, 2 minutes at 2700 rpm, 4 minutes at 2400 rpm, 4 minutes at 2700 rpm, 3 minutes at 3000 rpm, and 36-second deceleration, and stopped. At the end of the centrifugation process, 3 blood fractions were identified (Fig. 1):

1. The upper most liquid phase of plasma termed as platelet-poor plasma (PPP)
2. The second layer which is most important and is a solid layer of CGFs consisting of 3 fractions:
   - an upper white part (WP) of CGF
   - a red part (RP) at the lower end
   - a buffy coat at the interface between WP and RP
3. The third layer at the bottom consisting of red blood cells (RBCs)

For each test tube, the upper most PPP was carefully poured out, whereas the solid CGF and RBC were removed from the test tube using sterile tweezers and placed in a specific sterile dappen dish. The RBC layer was then cut off leaving the solid CGF sample. This process was repeated for each sample; the weight of each CGF sample was recorded and put into a separate test tube.

Round-up and β-Tricalcium Phosphate

Once the centrifugation was completed, 5 of the CGF samples from V1 and 5 of the CGF samples from V2 were mixed with β-TCP (Combioss, Silfradent srl) in a specific Round-up Machine (Silfradent srl). This device is for an intrinsic and extrinsic molecular blend, mixing without altering the geometric dimensions of autologous, heterologous, or synthetic materials. The system allows to obtain a homogenous mixture free from atmospheric contamination, in <16 seconds. Moreover, it is equipped with a decontamination cycle with UVC reflected light. Each experimental CGF sample, having had its whole weight recorded as mentioned above, was mixed with 1 mL of β-TCP (β-TCP ruCGFs). The CGF samples without β-TCP were also placed in the Round-up to act as controls (ruCGFs).

Both experimental (β-TCP ruCGF) and control (ruCGFs) CGF samples were placed into the Round-up device for 10 seconds. At the end of the process, each CGF sample was removed from the Round-up, picked up under a laminar flow cabinet and placed in separate wells of a 6-well plate, adding 3 mL/well of standard RPMI 1640-cell medium (Lonza, Verviers, Belgium) without growth supplements. In addition to the experimental and control samples, CGFs alone samples were also placed in wells without any mixing with β-TCP and any Round-up.

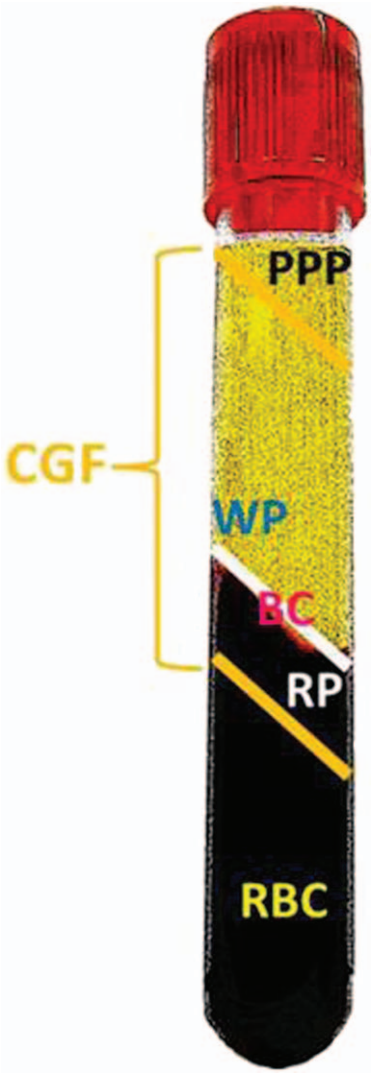

FIGURE 1. Blood sample after CGF centrifugation. Three layers are obtained: upper layer, PPP; middle layer, CGF consisting in 3 fractions: the upper WP, the downer RP and the middle BC; lower layer RBC. BC, buffy coat; CGF, concentrated growth factor; PPP, platelet-poor plasma; RBC, red blood cells; RP, red part; WP, white part.

Multiwell plates were then incubated at 37°C, 5% CO2, in a humidified atmosphere. A supernatant was then collected from the samples at a period interval of 5 hours, 1 day, 3 days, 6 days, and 8 days and centrifuged at 400 g for 10 minutes at room temperature. These supernatants were then divided into different aliquots and stored at −80°C for enzyme-linked immunosorbent assay (ELISA).

Cumulative Growth Factor Release

The quantification of growth factors was performed using ELISA kits according to the manufacturer's protocol (R&D Systems Inc, Minneapolis, MN). The total quantity of growth factors being investigated at different time points was checked and reported as mean value of all volunteers and expressed as total picograms.[37,46]

Statistical Analysis

One-way analysis of variance test corrected by Bonferroni was used for statistical analysis. A P value <0.05 was considered statistically significant. Results (Fig. 2) were expressed as mean ± standard error (SE).

RESULTS

The total quantity of growth factors obtained at fixed time points (5 hours, 1, 3, 6, and 8 days) with β-TCP ruCGFs or without β-TCP ruCGFs were compared. In addition, quantity of growth factors obtained for CGFs alone at different time points was also compared. In general, β-TCP ruCGFs show an increased release of BMP-2 and BMP-7, but it does not affect the TGF-β1 and IGF-1 release, when compared to ruCGFs and CGFs alone. There was statistically significant ($P < 0.05$) increased release of BMP-2 and BMP-7 from β-TCP ruCGFs when compared to CGFs alone at 5 hours, 1, 3, 6, and 8 days. When comparing release of BMP-2 from β-TCP CGFs with ruCGFs, there was an increased release which was not statistically significant ($P > 0.05$). However, for the release of BMP-7, there was a statistically significant ($P < 0.05$) increased release from β-TCP CGFs with ruCGFs at 3 days and 6 days.

As shown in the Figure 2A, the release of BMP-2 progressively increases in β-TCP CGFs, reaching the maximum accumulation at the 3rd day and after this it maintains a plateau, during all the experimental time. Also, in ruCGFs, BMP-2 reaches the maximum accumulation at the 3rd day, but decreases afterwards. On the contrary, in CGFs alone, the release of BMP-2 is lowest of the 3 samples, but it is statistically significant at 8 days compared to CGFs alone for 5 hours (Fig. 2A).

For BMP-7 (Fig. 2B), the release is higher in β-TCP CGFs, reaching the maximum accumulation at the 6th day and decreases after that. As stated above, there was a statistically significant ($P < 0.05$) increased release of BMP-7 from β-TCP CGFs at 3 and 6 days when compared to ruCGFs and at 5 hours, 1, 3, and 6 days when compared to CG alone. The release of BMP-7 from ruCGFs follows a similar pattern as from CGFs alone but with an increased quantity, reaching the maximum accumulation at the 8th day, that is, the last part of the experimental period (Fig. 2B). Similarly to BMP-2, also the release of BMP-7 from CGFs alone is statistically significant at 8 days compared to CG alone 5 hours (Fig. 2B).

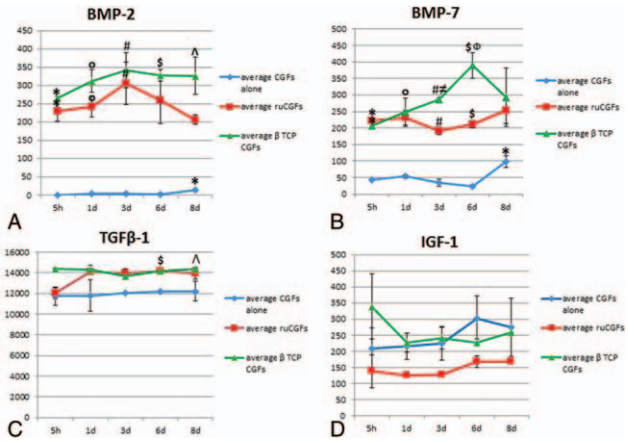

FIGURE 2. Cumulative growth factors released by CGF with β-TCP and Round-up, CGF with Round-up, and CGF alone (no Round-up and β-TCP) at 5 hours, 1 day, 3 days, 6 days, and 8 days of culture. The mean values of volunteers are plotted. The values are reported as total amount of growth factors released ± SE. $^{*}P < 0.05$ vs CGF alone 5 hours. $^{\circ}P < 0.05$ vs CGFs alone 1 day. $^{\#}P < 0.05$ vs CGF alone 3 days. $^{\$}P < 0.05$ vs CGF alone 6 days. $^{\Lambda}P < 0.05$ vs CGF alone 8 days. $^{\neq}P < 0.05$ vs ruCGF 3 days. $^{\Phi}P < 0.05$ vs ruCGF 6 days. β-TCP, β-tricalcium phosphate; BMP, bone morphogenetic protein; CGF, concentrated growth factor; IGF-1, insulin-like growth factor-1; TGF-β1, transforming growth factor-β1; ruCGFs,CGFs mixed with Round-up.

The release of TGF-β1 is increased in both β-TCP CGFs and ruCGFs, compared with CGFs alone (Fig. 2C). However, this increased release was statistically insignificant apart for day 6 and 8, when there was a statistically significant ($P < 0.05$) increased release of TGF-β1 from β-TCP ruCGFs and ruCGFs respect with CGFs alone (Fig. 2C).

Finally, as shown in Figure 2D, the release of IGF-1 showed a different trend as compared to the other growth factors. The release was highest from β-TCP ruCGFs at 5 hours; however, there was a sharp decline in its release at day 1 and 3, reaching to almost similar levels of CGFs alone. For the rest of time points, the release of IGF-1 was maximum in CGFs alone followed by β-TCP ruCGFs. In summary, it can be stated that BMP-2 and BMP-7 release significantly increases after mixing up with β–TCP in the Round-up device.

DISCUSSION

BMPs act as growth and differentiation factors and chemotactic agents. Moreover, BMPs can induce differentiation of mesenchymal progenitor cells into various cell types including chondroblasts and osteoblasts. This suggests that BMPs may be able to influence both direct and indirect bone formation. Also BMP-2 and BMP-7 play an important role in bone and cartilage development.[47] They are involved in the hedgehog pathway, TGF-β signalling pathway, and in cytokine-cytokine receptor interaction. They are involved also in cardiac cell differentiation and epithelial to mesenchymal transition. BMP-2 and BMP-7 are osteoinductive BMPs: they have been demonstrated to potently induce osteoblast differentiation in a variety of cell types, promoting osteogenesis and so bone and periodontal regeneration.[48–50] Hakki et al[51] showed that BMP-2, BMP-6, and BMP-7 differently regulate osteogenic differentiation of human periodontal ligament stem cells and could be a promising strategy for bone tissue engineering. Sun et al[52] showed that BMP-2 may facilitate the osteogenetic differentiation of mesenchymal stem cells. Moreover, other evidences[53,54] suggest that the absorbable β-TCP block may be an effective bioceramic for bone induction delivery of BMP-2 to the site of action. Other studies[55,56] indicate that the use of β-TCP in combination with BMPs (especially BMP-2) significantly increased bone formation. These data could be supported also by our findings which have shown that the combination of CGFs, β-TCP, and Round-up increases the release of BMP-2 and BMP-7. A possible explanation for this is that the process of Round-up crushes the CGF, making some growth factors (like BMP-2 and BMP-7) immediately available. These are then released more quickly and in greater quantities.

Another possible reason for increased release of BMP-2 and BMP-7 could be because of the presence of Ca^{2+} in β-TCP. A large body of evidence supports a general role for Ca^{2+} in platelets activation and degranulation.[57] In fact, elevation of Ca^{2+} amplifies platelet activation, via stimulation of several target proteins, for example, Ca^{2+}-dependent activation of myosin light chain kinase and protein kinase C mediate cytoskeletal rearrangements associated with shape change and platelet granule release, respectively.[58] In addition, elevated extracellular Ca^{2+} has been implicated in osteogenesis by stimulating the proliferation and differentiation of osteoblasts. It was shown that elevated Ca^{2+} increases BMP-2 gene expression in human dental pulp cells.[59] The increase was modulated not only at a transcriptional level but also at a post-transcriptional level because treatment with Ca^{2+} increased the stability of BMP-2. A similar increase in BMP-2 mRNA level was observed in other human mesenchymal cells from oral tissues, periodontal ligament cells and gingival fibroblasts. Hence, these findings could explain the increase in BMP-2 and BMP-7 release, in presence of β-TCP.

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