BONE HEALING STIMULATION BY PLATELET-RICH AUTOGENOUS PLASMA. AN EXPERIMENTAL STUDY IN RABBITS

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SUMMARY

The autogenous blood plasma with high platelet concentration obtained through centrifugation (platelet-rich plasma, or PRP) has been used in clinical practice to stimulate bone healing in a number of situations, allegedly because of its ability to carry a high concentration of platelet-derived and β-transformer growth factors, which are well known to stimulate different tissues growth and repair. In the present study, PRP was used to repair a half-thick, 2-cm long segmental diaphyseal bone gap produced on New Zealand rabbits' radius. Periosteum was dried at the circumference of the gap site and the spinal cord cavity was sealed with bony wax in all animals in order to block the entrance of repairing cells other than the ones from the bone itself, but from surrounding tissues. Three groups of 15 animals each were designed, according to the procedure performed: 1) gap left empty; 2) gap filled with PRP; and 3) gap filled with an inert material (Gelfoam®). In each group, the animals were deployed into three subgroups according to postoperative follow-up period, of 4, 8, and 12 weeks, respectively, after which animals were sacrificed and the radius was dried for histological study purposes. X-rays and scintiscan were taken within 4 weeks intervals, starting from the fourth postoperative week. Full healing and remodeling were seen in group 2 as soon as the 8th postoperative week, while in groups 1 and 3, that process was only partial at the 12th week. Tecnetium uptake was increased in all groups, remaining as such throughout the whole follow-up period in groups 1 and 3, but showing reduction between the 8th and 12th week in group 2, accompanying remodeling process, with significant differences between groups ($p<0.05$).

Keywords: *Bone and bones; Blood platelets; Radionuclide imaging*

INTRODUCTION

Spontaneous repair of different tissues in human body, including bone, is mediated by different growth factors, in a process started by blood clog formation and continued by subsequent platelet degranulation, which releases growth factors [1,2,3]. Many growth factors have been implied on bone repair process: platelet-derived growth factors derived (PDGFs), vascular endothelium growth factor (VEGF), transforming growth factors α and β (TGF-α and TGF-β), acid and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), insulin-like growth factors I and II (IGF-I and IGF-II), cement-derived growth factor (CGF), parathyroid hormone related proteins (PTHrP), and bone morphogenetic protein 1 to 12 (BMPs 1-12,)[4,5]. Some of these growth factors, such as PDGFs (aa, bb and ab), TGFs β1 and β2, VEGF and EGF are inside platelets' α granules[6].

Although produced by platelets, PDGFs are not usually detected on blood plasma and its concentration drops fast (within less than two minutes) to almost zero when directly injected into blood stream due to its hydrophobic and highly cationic nature [7]. On the other hand, PDGFs are very easy to obtain, because its concentration theoretically increases with platelets concentration by centrifugation, in the so-called platelet-rich plasma (PRP). A normal blood clot contains about 95% of red blood cells, 5% platelets and less than 1% of leukocytes; in PRP, the ratio between red blood cells and platelets is the contrary, with the latter occupying approximately 95% of total volume. In fact, PRP preparation process consists of platelets segregation, of which concentrations may increase up to six times / volume, compared to normal blood.

PRP has been already used in many medical areas (reconstructive plastic surgery, ENT) and in dental care to produce homeostasis, stimulate soft tissues and bone healing, skin grafts adherence, bone grafts union, and implants fixation on skull and face flat bones [5,8,9,10], but, despite of the evidences showing that the PRP may be useful in those situations, there is no unanimity about its role in bone healing [11,12]. Furthermore, there are few reports about beneficial effects on locomotive apparatus' long bones healing, although "substantial bone neoformation", increased vascular invasion, and better bone graft remodeling have been shown in metatarsal bones of sheep, in which a critical defect was produced, filled with a combination of homologous graft, stem cells and PRP [13].

Considering the great clinical application potential of PRP, stronger evidences showing that it really stimulates bone

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neoformation and healing are required. The present study has as an objective to investigate PRP's role on the healing process of a half-circumference longitudinal diaphyseal bone failure produced on rabbits' radius.

MATERIALS AND METHODS

The study design was approved by the Committee on Ethics in Animal Experiments, FMRP-USP. Forty-five New Zealand 8 - 10 week-old male rabbits were used, and divided into three groups (1, 2 and 3) of 15 animals each, according to the procedure performed. Animals in each group were distributed into three subgroups of five animals each, according to postoperative follow-up period, of four (subgroup a), eight (subgroup b) and 12 weeks (subgroup c). All animals were kept in individual cages during the whole experimental period, under strict hygienic conditions and fed with standard ration for rabbits and water ad libitum.

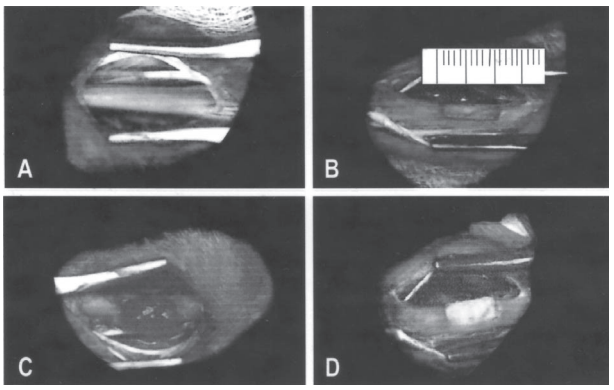

Figure 1 - *Surgery details: the 4-cm long segment of exposed radius, with dried periosteum (A); 2 cm long and half diameter bone gap resulting from resection osteotomy (B); gap filled with PRP (C) and gap filled with inert material (D).*

Surgical procedure

Under general anesthesia (Ketamin hydrochloride, 35 mg/kg*; Xylazine, 9 mg/kg**) administered intramuscularly with a tourniquet applied on humeral segment of anterior member, the antebrachial segment was routinely prepared for surgery (trichotomy, antisepsis with 20% iodinated alcohol, surgical drapes involving the operating field). Radius was exposed through a longitudinal straight incision at antebrachial segment's medial edge. The space between extensor and flexor muscles groups was dissected, providing a wide view of radius, of which periosteum was fully dissected in a 4-cm long segment. With a delicate motor saw, necessary longitudinal and transversal osteotomies were performed to remove a half-circumferential, 2 cm long window of the bone, leaving a corresponding bone failure open. Medullary channel was blocked with bone wax (Ethicon®) above and below failure, which was left empty in group 1, filled with autologous PRP in group 2, and filled with an inert material (Gelfoam®) in group 3 (Figure 1).

Autogenous PRP preparation

With the animal under anesthesia, a 4.5-ml blood sample was collected by means of cardiac puncture, which was placed in an appropriate assay tube and centrifuged during 15 minutes at 1800 rpm. Heavier red blood cells were collected deep in the tube, while plasma remained on top, as usual. From the bottom to the top of the tube, the first plasmatic layer (about 100 μl), including a layer of about 1 mm thick still containing red blood cells, was the platelet-very rich plasma (*pvrp*); the second layer, indivisible from the first one (about 500 μl) was the platelet-rich plasma (*prp*), while the third layer, similarly indivisible from the second one (about 500 μl), was the plasma with moderate platelet content (*pmp*). A fourth layer was constituted of poor platelet content (*ppp*). The first and second layers occupied a 13-mm long segment of the tube, just above red blood cells layer, and were aspirated together with the aid of a pipette, thus constituting the PRP, which was deposited in a second tube and added by 10 μl of 10% calcium chloride solution for inducing coagulation. Once coagulated, PRP was ready to fill bone failures in group 2. The whole PRP preparation procedure was performed under strictly sterile conditions.

Platelets were counted in whole blood and in PRP of each animal in order to assure that the strength achieved by centrifugation was within requirements for stimulation. In whole blood, the mean strength was 324,000 platelets /mm^3 (range: 248,000 – 634,000 platelets /mm^3) and in PRP, that was 1,239,000 platelets /mm^3 (range: 740,000 – 2,250,000 platelets /mm^3), meaning a four-fold average increase.

Postoperative evaluation

This was constitute of conventional X-ray images, technetium scintiscan, and histological analysis of operated bone, dried after the animals were sacrificed within four, eight and 12 weeks, according to the subgroup in each group.

Conventional X-ray images: the radial segment of the anterior limb was rigorously maintained at established positions to enable comparisons, and X-ray findings were graded according to their appearance, namely: 0. total absence of healing; 1. uneven bone neoformation (undetermined healing signs); 2. bone sclerosis and response; 3. partial healing, and; 4. total healing/ remodeling.

Bone scintiscan: promptly obtained (balance phase) and three hours (impregnation phase) after endovenous injection of a technetium formula (Tc99m methylene-diphosphonate, 8 μCu), soon after conventional X-ray images were taken. The results were recorded as counts per minute and the quotient of values recorded for right radius (operated) and left radius (intact) was calculated, and named as activity quotient (R/L quotient).

Histological studies: the animals were killed four, eight and 12 weeks postoperatively, according to the subgroup in each group, with an endovenous injection of a massive dosage of general anesthetic, causing a quick and painless death. The right radius was removed by disarticulation at elbow level, and thoroughly cleared of surrounding soft parts and sunk in 10% formalin during five days, for fixation; then, it was decalcified in a water solution of 5% nitric acid during additional 5 days. Once decalcified, both epiphyses were dried, leaving only the intermediate segment with operated site, which was divided into three segments (proximal, intermediate, and distal) of similar length. Each of those segments were included in par-

* Dopalen®; ** Rompun®

affin blocks, by which 6-μm thick histological sections were obtained, mounted in a slide and stained with hematoxylin-eosin (HE). Sections were examined with the aid of a light microscope (Zeiss Axiophoto) mounted with a video camera and attached to a computer for images capture, storage and handling. Results concerning bone failure repair were graded according to the amount of neoformed bone tissue, as follows: 0. absence of osteoclasts and osteoblasts; 1. minimum amount of neoformed bone; 2. sparse isles of osteoblasts grouping; 3. more organized osteoblasts grouping and young bone trabeculas; 4. bone trabeculas interposed with medullary spaces.

Data concerning X-ray, scintiscan and histological analyses were submitted to statistical analysis by Student's t-test and by variance analysis (ANOVA), at a significance level of 5% ($p \leq 0.05$).

RESULTS

Anesthetic and surgical procedures, as well as the confinement in cages were well tolerated by all animals. No fracture occurred, but some curving of the operated radius was observed in some animals from all groups.

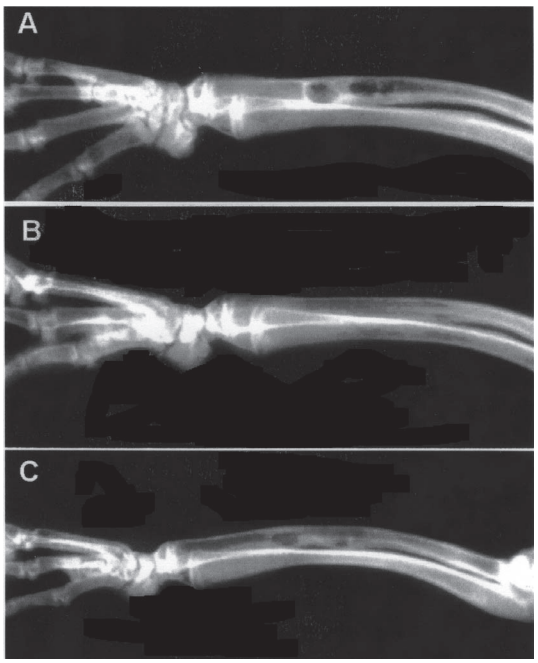

Figure 2 - *X-ray appearance of operated site after 12 weeks: incomplete gap healing in groups 1 (A) and 3 (C), with reactive neoformation in the latter. Full healing in group 2 (B).*

	Postoperative Period (weeks)		
Group	4	8	12
1	1.18 (1.05-1.30)	1.25 (1.12-1.45)	1.28 (1.16-1.48)
2	1.61 (1.47-1.68)	1.54 (1.43-1.62)	1.28 (1.21-1.35)
3	1.20 (1.09-1.33)	1.29 (1.19-1.36)	1.31 (1.21-1.41)

Table 1 - *Distribution of activity quotients (average and range), according to groups and follow-up periods.*

X-ray evaluation

The results graded as described above allowed for a semi-quantitative evaluation and corresponding statistical analysis. Uneven bone neoformation, characterized by areas of apparent young bony callus interposed with areas apparently empty was observed in group 1 within eight (grade 1) and 12 (grade 2) weeks. In group 2, the same kind of bone neoformation (grade 2) could be seen within four weeks, the full gap healing (grade 4) being evident within 8 weeks, period in which the gap had virtually disappeared, thank to bone remodeling, which became more evident within 12 weeks, when recognition of the surgical site was already impossible for the majority of cases. In group 3, some bone neoformation (grades 1 and 2) was observed inside the gap and in surrounding areas within 8 and 12 weeks, but the overall appearance was very similar to group 1 (Figure 2). Differences observed in X-ray findings were significant between group 2 and groups 1 and 3 ($p<0.05$), but not between groups 1 and 3 ($p>0.05$).

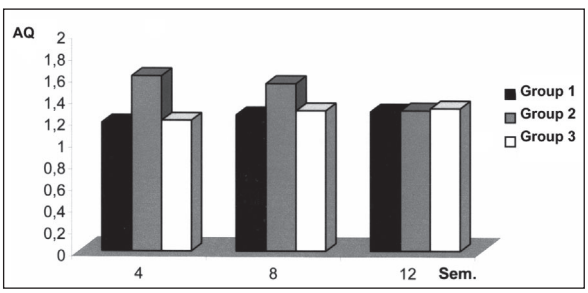

Figure 3 - *Graph for activity quotients (AQ) behavior in the different groups, showing that the isotope uptake was still increasing in groups 1 and 3, as a result of an active bone neoformation process, while in group 2, it was in an overt decrease, meaning that a healed gap was already under remodeling process.*

Scintiscan evaluation

Count values for radioisotope uptake were not even, both in the operated limb and the intact one among animals of all groups and subgroups. In fact, they substantially differed, but were consistently higher in the operated limb, as evidenced by activity quotient. Slightly increased activity quotients, ranging from 1.1 to 1.07, but showing a likelihood to decrease with time, were observed in all subgroups at balance phase, with four, eight and 12 weeks, indicating the occurrence of an accommodation of vascular disarrangement caused by surgical procedure. Differences between groups were not significant in this phase in none of the periods (>0.05).

At impregnation late phase, the average activity quotient progressively increased in groups 1 and 3, respectively: 1.18 and 1.2 with four, 1.25 and 1.29 with eight, and 1.28 and 1.31 with 12 weeks, with no significant differences between subgroups and groups (>0.05). For group 2, the average activity quotient was 1.61 with four weeks, being reduced to 1.54 with eight, and to 1.28 with 12 weeks, with differences between periods being significant ($p<0.05$). But, differences between group 2 and groups 1 and 3 were significant in four and eight weeks ($p<0.05$), but not in 12 weeks ($p>0.05$) (Table 1, Figure 3).

Gross evaluation

Almost no tissue reaction was evident in tissues around the gap in groups 1 and 2, except for group 3, in which some thickening and muscle fibrosis were noticed. The gap itself was filled with bony callus in group 2 within four weeks, so remaining within eight and 12 weeks. In group 1, within four weeks, virtually no bony callus existed

in the gap, which was filled with fibrous tissue, so remaining during subsequent periods. In group 3, it seemed to have some bone tissue inside the gap from the eighth week on, becoming more evident within 12 weeks. Approximately half the amount of radius was slightly curved, but none of them was evidently fractured.

Histological studies

In group 1, within four weeks, the gap was virtually empty (grade 0) and the medullary space seemed to be filled with adipous tissue with sparse hematogenic cells. In eight weeks, some osteoblasts (grade 1) could already be seen, especially at gap periphery, in spite of a very poor osteogenic activity. A similar appearance was observed within 12 weeks (grades 1 and 2).

In group 2, within four weeks, many osteoblasts groupings, already outlining young trabeculas, and neoformed vessels occupied the gap (grade 3), but in a higher concentration at the periphery. Within eight weeks, young bone trabeculas increased in number and were more evident (grades 3 and 4), with many osteoblasts sparsely distributed inside the new calcified bone matrix. Within 12 weeks, bone neoformation was more compact, with bone trabeculas more organized and calcified, sometimes surrounded by osteoclasts, evidencing remodeling process (grade 4). Bone trabeculas outlined medullary spaces filled by abundant hematogenic tissue, similar to a normal bone.

In group 3, within four weeks, no osteoblastic activity was evident inside the gap (grade 0), with medullary space showing adipous cells, few blood vessels and amorphous material, reminiscent of the material used to fill the gap. Within eight weeks, some few osteoblasts groupings (grade 1) could be seen on gap's periphery, as if surrounding the amorphous material already partially reabsorbed, but still present. Within 12 weeks, the amorphous material had almost completely disappeared; around its remittances, some osteoblast groupings and few neoformed blood vessels could be noticed (grade 2) (Figure 4).

DISCUSSION

Despite of the great advancements of modern orthopaedic surgery, the surgical repair of bone gaps resulting from trauma or other causes is still a challenging issue for orthopaedic doctors, especially when long bones' diaphysis is involved. Conventional, and, sometimes, vascularized autologous bone grafts are the procedures most commonly performed, but the sources of autologous grafts are not infinite, thus the orthopaedic doctor may have to use other materials or techniques to solve complex cases requiring multiple grafting procedures. Homologous grafts are an alternative, but their use depends on the existence of a bone base, available only in large specialized centers; furthermore, these can represent an additional threat to patients due to transmissible diseases such as AIDS and type C Hepatitis. Thus, the search for new alternatives for autogenous tissue transplants is highly valuable, and this is where bone neoformation stimulus with PDGFs fits.

Morphogenesis is a complex process by which different tissues of the human body, including bone, are formed, from multipotential mesenchymal cells, mediated by different growth factors. These are polypeptides which can potentially promote cells and tissues differentiation and growth, mediating mitoses, chemotaxis and metabolism; they also stimulate and regulate healing process of different tissues (Marx, 1996). Each growth factor stimulates the development of blood cells, being responsible for many tissues growth in serum-dependent cultures[7]. They also seem to be responsible for the beginning of spontaneous repair in connective tissues, including bone, increasing the number of mitoses (repairing cells), angiogenesis (new blood vessels formation) and macrophagocytic activity (débridement).

As PDGFs are highly unstable and don't last long if free in blood stream, PRP is theoretically an adequate vector to increase its concentration in injured tissues. The slow release of PDGFs by platelets degranulation would provide enough concentration to trigger tissue growth stimulus. Despite some controversial data, PRP beneficial effects have been shown, especially in bucomaxillofacial surgery and dental areas, but the same benefits with its use alone or combined with other materials for stimulating locomotive apparatus' long bones repair have not been sufficiently shown yet. Indeed, considering that long and flat bones result from different kinds of ossification, would it be possible that the previous respond as well as the latter, such as maxilla and mandible, to PRP?

The results achieved in this investigation indicate that PRP really stimulates a favorable reaction of long bones. The X-ray evaluation at 4-week intervals showed that bone gap was already healed in group 2 (filled with PRP) long before groups 1 (left empty) and 3 (filled with inert material), and was already being remodeled when, in other groups, it was still healing, within eight and 12 weeks. This fact was corroborated by scintiscan analysis, which showed that bone neoformation was strong in four weeks, in group 2, with isotope uptake at operated bone around 60% stronger than in the intact bone, slightly reducing within eight weeks (54%) and

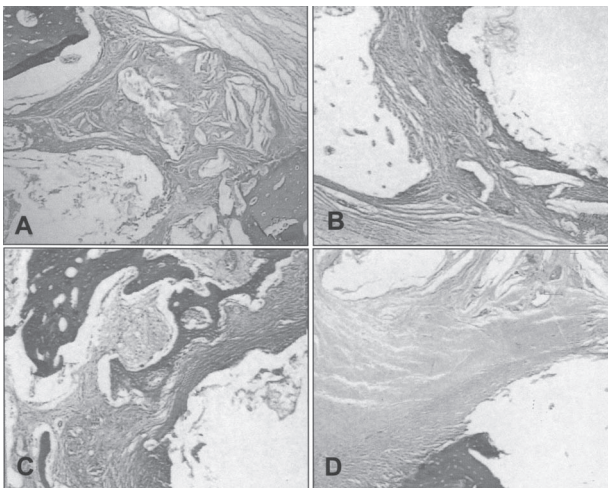

Figure 4 - *Photomicrographs of histological sections (hematoxylin-eosin) of specimens from different groups, 12 weeks postoperatively. Endosteal inflammatory response and fibrosis in group 1 (A, 100x); a new bone trabecula being formed, with indistinctive young osteoblasts in group 2 (B, 40x); mature bone trabecula with high osteoblastic activity in group 2 (C, 100x); fibrous tissue trespassing medullary channel in group 3 (40x).*

intensively within 12 weeks (28%), a typical behavior of a remodeling bone [14]. On the other hand, in groups 1 and 3, the isotope uptake smoothly and gradually increased during the same period, meaning that the body was still struggling to heal the gap. The histological evaluation confirmed that bone neoformation was stronger and more advanced in group 2 than in controls, with mature bone trabeculas already present within four weeks, with differentiation into cortical bone within eight weeks, while in groups 1 and 3, bone neoformation was still starting at those periods.

PRP preparation itself is very simple, at the reach of any surgeon having the infrastructure of a blood storage center and a hematologist to handle a patient's blood. As PRP is an autogenous preparation, there is no contamination risk for the patient, including infections at surgical site, provided that sterilized tubes and instruments, as well as careful techniques are used. Furthermore, PRP may be used in combination with autogenous or homologous bone grafts, or any other replacement material. The normal concentration of platelets in rabbits' whole blood is, in average, about 450,000 / mm^3, very close to values measured before PRP preparation in this investigation. The centrifugation method used here was able to multiply that concentration by four, which is within the range regarded as appropriate for stimulating bone neoformation, so that the same procedure could be eventually adopted in human clinical situations.

In conclusion, PRP really stimulates healing process in locomotive apparatus' long bones and seems to have an unlimited potential for clinical application in any situation requiring massive bone grafting or repeated grafting procedures in human beings, with the advantage that red blood cells can be returned to the patient, as poultice. By the way, there are some news telling us about the use of PRP to fill bone gaps in human beings, such as, for example, the expanded acetabulum, combined with autogenous or homologous bone graft in hip prosthesis review surgeries and others*.

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