Platelet-rich plasma in bone repair of irradiated tibiae of Wistar rats[1]

Plasma rico em plaquetas na reparação óssea em tíbias irradiadas de ratos Wistar

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ABSTRACT
Purpose: To evaluate the influence of PRP addition on bone repair of circular defects created in irradiated tibiae of rats by histometric analysis. **Methods**: Sixty male Wistar rats had the right tibiae irradiated with 30 Gy. After 30 days monocortical defects were created and platelet-rich plasma was applied in 30 rats. In the control group defects were created but not filled. The animals were desanguinated after 4, 7, 14, 21, 56 and 84 days and the tibiae removed for histological processing. **Results**: There was a tendency in the PRP group to increased bone neoformation from 14-days to 84-days; in the control group increased bone neoformation was not seen after 21 days or later. **Conclusion**: The addition of platelet-rich plasma had a beneficial effect in the initial cellular regeneration period and enhanced bone formation in later periods when compared to control.
Key words: Platelet-Rich Plasma. Radiation Injuries, Experimental. Bone Regeneration.

RESUMO
Objetivo: Avaliar histometricamente a influência do PRP na reparação óssea de defeitos circulares criados em tíbia irradiada de ratos Wistar. **Métodos**: Sessenta ratos machos tiveram a tíbia direita irradiada com 30Gy. Após 30 dias, defeitos ósseos monocorticais foram criados e PRP foi adicionado em 30 ratos. No grupo controle os defeitos foram criados, mas não preenchidos. Os animais foram sacrificados em 4, 7, 14, 21, 56 e 84 dias e a tíbia removida para processamento histológico. **Resultados**: Houve uma tendência do grupo PRP mostrar uma neoformação óssea significativamente maior nos períodos de 14 a 84 dias; no grupo controle o aumento da reparação óssea não se manteve após 21 dias. **Conclusão**: A adição de PRP mostrou-se benéfica no período inicial de reparação celular e em períodos seqüentes foi estímulo à quantidade neoformação óssea, quando comparado ao controle.
Descritores: Plasma Rico em Plaquetas. Lesões Experimentais por Radiação. Regeneração Óssea.

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Introduction

Resection of malignant tumors, especially in the head and neck region, necessities reconstruction to give back to patient the possibility to speak, swallow, and maintenance of the respiratory pathways, and facial aesthetics. However, the results might be limited due to the damages inflicted on the tissues by the radiotherapy. The lower jaw is one of the bones more frequently affected by the radiation and complications can be seen years after the radiation, varying from the simple bone exposure to extensive bone necrosis accompanied by severe pain. The incidence of mandibular osteoradionecrosis in patients with cancer of head and neck vary in the literature from 0,4% to 56%, and though it typically takes place in the first three years after the radiotherapy, patients present a constant risk later on[1-3]. The irradiated bone is characterized by the absence of osteoclasts which induces delay in bone resorption; micro-fractures containing fibrous tissue but without formation of callus can further be seen; and three times more incomplete osteons are observed and intra-bone ischemia, multiple emboli, extensive tissue hypoxia and cellular secondary destruction has been observed[1].

The use of platelet rich plasma in bone regeneration was first used in order to aggregate particulate autogenous bone in mandibular reconstruction. From that time, the potential proliferative effect of platelet rich plasma (PRP) has been explored in craniofacial reconstruction, especially in oral rehabilitation with

dental implants, and with several graft materials.

Platelet rich plasma used in maxillofacial surgery seems to increase the speed of regeneration when mixed with bone grafts. The blood platelets, cytoplasmic fragments of megakaryocytes, secrete proteins that begin tissue regeneration, such as platelet-derived growth factor (PGDF), transforming growth factor alpha (TGF-alfa) and beta (TGF-beta), epidermal growth (EGF) and vascular endothelial growth factor (VEGF)[4]. In the plasma the mean normal concentration of platelet is 150,000 to 400,000/mm3 of blood and, after centrifugation, it can be increased in up to 338 %[5].

In the search of ideal therapies with growth factors that could provide improvement of the regenerative process of bone tissue affected by radiation, it seems valuable to study the applicability of the PRP, a material with osteoinductive, osteogenic and osteoconductive properties, from easy accomplishment and availability, immunologically inert and economically accessible.

Purpose

The aim of this experimental study was to evaluate the effect of platelet rich plasma in the bone regeneration of bone defects created in rat tibiae previously radiated with doses of 30Gy, and further to establish a comparative analysis with a radiated control group.

Methods

This experiment was approved by the ethics committee of UNIFESP - Federal University of Sao Paulo and followed the CIOMS (Council for International Organization of Medical Sciences) ethical code for animal experimentation.

Sixty (60) male Wistar rats, between 90 and 120 days of age and average weight of 450g constituted the sample of this study. cycle). They had *ad libitum* access to acidified drinking water (pH = 2,8-3,1) and a standard laboratory rat food pellet diet (20g/day). The animals were maintained in sterile individual polycarbonate cages.

Anesthesia

General anesthesia was induced, for both radiation and surgical procedures, by intraperitoneal injection of xilasin 2% (0,1ml/kg) and ketamine 10% (1ml/kg).

Radiation procedure

The radiation of the animals followed the protocol of Ohrnell *et al.*[6]. In rat, the application of 30Gy caused a significant reduction of the bone regeneration capacity but acute effects that were well supported by the animal. When a single dose of 30Gy was compared to clinical practice it would correspond to approximately 50-70Gy applied in fractioned radiotherapy[6].

The radiation procedure was carried out 30 days before the surgery. After anesthesia, 10 to 14 animals were simultaneously immobilized in a transparent acrylic plate. The right tibiae were put in a field of 20 X 20 cm size and 80cm source-skin distance and immobilized with a plastic fastener. The left paws and tails were fixed and put out of the field of radiation.

Before radiation, the animals were subjected to a digital simulator of radiation, to guarantee a homogeneous dose of radiation. In the sequence, the animals were irradiated with a single dose of 30Gy in a Cobalt 60 equipment.

PRP preparation

Two animals, anaesthetized and irradiated at same time of the of the experimental animals group, were sacrificed to get PRP sufficient for ten surgical procedures. An aorta heart puncture with complete exsanguination was performed using a 10ml sterile disposable syringe. Approximately 20ml of blood was collected and immediately transferred to sterile tubes with 0.5ml of 3.2% sodium citrate.

The blood was first centrifuged at 200G for 10min to separate plasma from red cells. With a disposable syringe, the whole portion of plasma in the superior part of the tube was drawn off and transferred to "dry" tubes. The second centrifugation, carried out at 200G during 15min, separated platelets in poor, average and rich phases. The two superior thirds of the platelet poor plasma were collected and dispensed. The inferior third, platelet rich plasma, was transferred to a sterile Petri plate. The quantity of PRP obtained was approximately 10 to 15% of the total volume of blood. The activator 10% calcium chloride was added in a ratio of 1:20 for total volume of PRP.

Platelet counts were performed to calculate the PRP concentrate, which should be around 400% of the peripheral blood platelet count or at least a concentration of 1.000.000 platelet/microliter in a volume of 5ml.

Surgical procedure

The animals were separated in two groups of 30 animals. The experimental group received the PRP graft in the bone defect in the tibiae, while in the control group was carried out only the bone defect.

The surgical procedures were performed 30 days after the radiation, given that the acute local side effects (epithelial desquamation, erythema and loss of hair) and systemic symptoms (illness and fatigue) after a dose of 30Gy were reverted in the first three weeks[6] and the interval of time between the radiation and the surgery does not influence the bone regeneration[7].

Both right and left sides were operated to assure similar morbidity and so to reduce the possibility of the animal to use more the non irradiated side (left) in detriment of the experimental irradiated side (right).

The animals were anaesthetized, and both legs were shaved and washed with iodine-povidine. The operation was effectuated in sterile environment and with a gentle surgical technique. A longitudinal linear incision of approximately 2cm in the medial region of the tibiae was made to expose the subcutaneous tissue and the musculature. The periosteum was exposed and incised, exposing the bone tissue of the medial portion of the tibiae metaphysis.

The circular monocortical defects of 3mm of diameter were created with trephine burs (FTR02 - Sin, Brazil) under profuse and constant irrigation with saline sterile solution to avoid thermal damage to the tissues (Figure 1).

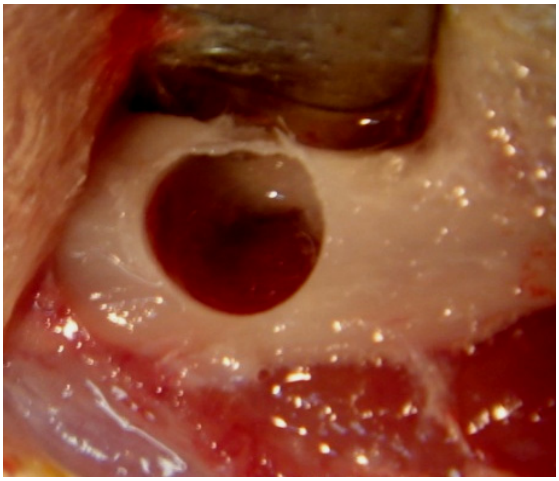

FIGURE 1 - Bone defect

In the experimental group, the resultant bone defect was filled up to superior cortical limit by PRP, while in the control group there were no grafts.

The wound was closed in layers; the periosteum and muscular tissue were sutured with resorbable suture (Vicryl rapide 4-0) and the skin was sutured with nylon 4-0 suture.

A dose of 16.000UI of antibiotic benzatil-penicillin was given by intramuscular injection after surgery; paracetamol in the dose of 500mg/500ml of water to drink was administered to the animals in the first two post-operative days.

Histological examination

In the periods previously established at 4, 7, 14, 21, 56 and 84 days after surgery, subgroups of five animals each were sacrificed by an overdose of anesthetic.

Skin around bone was carefully removed and the tibiae were cut off with a bone saw. The pieces obtained were conditioned in labeled containers with 10% formaldehyde solution for at least 72 hours. After fixation the muscular tissues were removed and the circular defects with a 3mm margin of surrounding bone were sawn transversely. The specimens were decalcified in a 10% EDTA solution during 24 to 36 hours, and then washed in running water for 24 hours. Dehydration was prosecuted in successive passages in alcoholic and xylol solutions in automatic equipment. Specimens were then embedded in paraffin, cut in 3 to 7 um thickness cross sections and stained with hematoxyline-eosin and Masson trichrome stain.

Sections were examined in a light microscope, connected to a camera and microcomputer Pentium 233Mhz equipped with IMAGE-PRO ® PLUS - Version 3.0 software for morphometrical measurements. The images were manipulated in Adobe Photoshop 6.0 with magic wand and rubber tools to delimit only the bone tissue. The bone neoformation area was measured in micrometers using Image J 1.38x software.

Statistical methods

The statistical proceedings were carried out with Statistical Package Social Sciences (SPSS) version 11.0 Windows. ANOVA and Tukey were used for all comparisons between groups and periods. The level of significance alfa was set at 5%.

Results

Table 1 and Figure 2 show the measured bone neoformation area for control group (CTR) and experimental group (PRP) according to the time period. It is possible to notice that the bone area in CTR group seems to increase up to 21 days, but decreased in the subsequent periods. In PRP group the neoformation was progressively increasing during a period of 14 days and stabilized after 21 days. The variability of the data in the group CTR also seems bigger than in the group PRP.

The results of the comparisons (ANOVA) between the CTR and PRP groups in each period revealed statistically significant differences. The PRP group presented significantly bigger bone neoformation than the control group in all the periods, except to 7 days. Since the measures in the 4 days period presented null variability, the groups were not compared between them.

TABLE 1 – Mean and standard deviation for bone neoformation area. Comparison between CTR and PRP groups for each period

Period	Group	Mean	SD	ANOVA	p-value
4	CTR	0,0	0,0	—	—
	PRP	0,0	0,0		
7	CTR	4977,1	14931,3	CTR = PRP	0,247
	PRP	22781,3	40638,8		
14	CTR	21225,4	24018,1	CTR < PRP	<0,001
	PRP	232909,8	51281,3		
21	CTR	126942,2	101958,4	CTR < PRP	0,012
	PRP	237267,9	28240,4		
56	CTR	53892,6	90457,2	CTR < PRP	<0,001
	PRP	238381,6	40296,6		
84	CTR	121003,8	111498,7	CTR < PRP	0,005
	PRP	264054,9	31758,2		

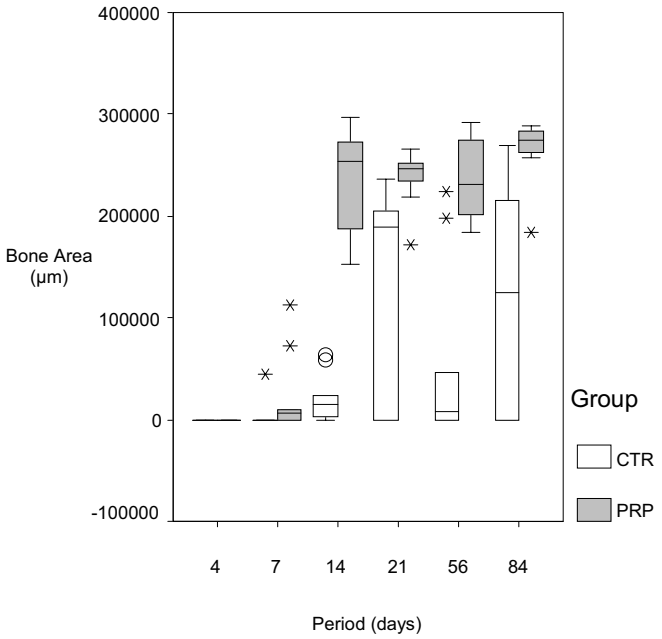

FIGURE 2 – Boxplot. Comparison between CTR and PRP groups for each period

The comparisons between the periods for CTR and PRP groups are respectively presented in Table 2. In CTR group only the bone neoformation of the period of 21 days was superior to that of the period of 7 days. So the result of the comparisons suggests that the CTR group did not show significant bone neoformation during the 84 days of experiment.

In the PRP group, however, all the periods after 14 days were superior to the period of 7 days. Nevertheless, after 14 days the quantity of bone was not different between the periods, suggesting that the effect of the PRP is more present in the first 14 days.

TABLE 2 - Comparison between the periods for CTR and PRP groups

Compared periods		CTR	PRP
7	14	7 = 14 (p=0,453)	7 < 14 (p<0,001)
7	21	7 < 21 (p=0,042)	7 < 21 (p<0,001)
7	56	7 = 56 (p=0,534)	7 < 56 (p<0,001)
7	84	7 = 84 (p=0,079)	7 < 84 (p<0,001)
14	21	14 = 21 (p=0,082)	14 = 21 (p=0,999)
14	56	14 = 56 (p=0,828)	14 = 56 (p=0,999)
14	84	14 = 84 (p=0,148)	14 = 84 (p=0,551)
21	56	21 = 56 (p=0,514)	21 = 56 (p>0,999)
21	84	21 = 84 (p>0,999)	21 = 84 (p=0,361)
56	84	56 = 84 (p=0,635)	56 = 84 (p=0,577)

Figure 3 to 8 show histological results for for CTR and PRP groups in each experimental period.

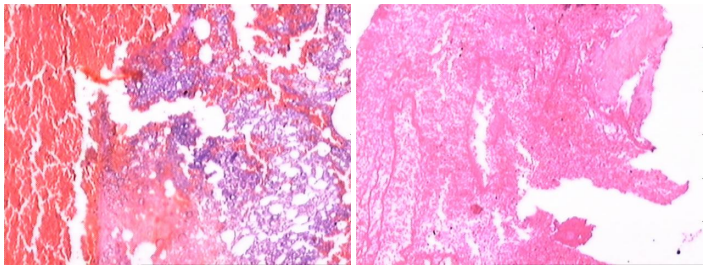

FIGURE 3 – Control x PRP. Period of 4 days (100x)

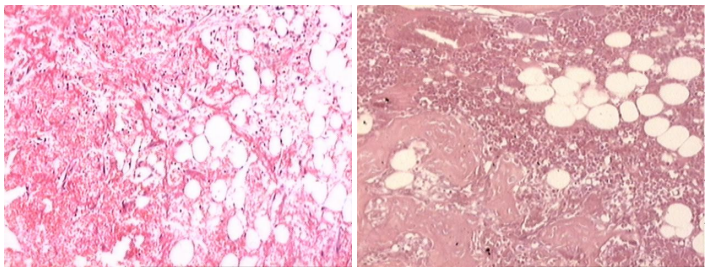

FIGURE 4 – Control x PRP. Period of 7 days (100x)

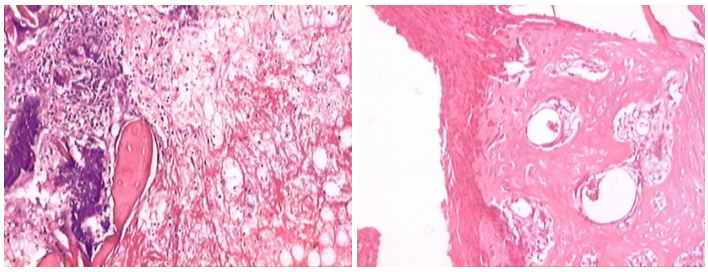

FIGURE 5 – Control x PRP. Period of 14 days (100x)

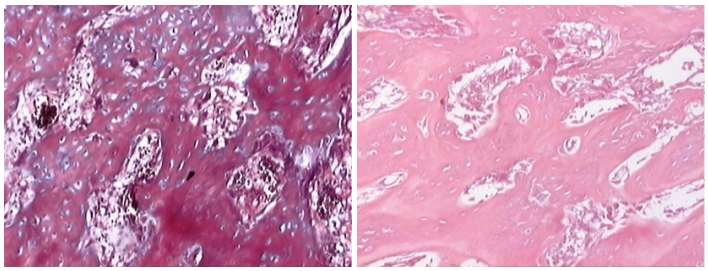

FIGURE 6 – Control x PRP. Period of 21 days (100x)

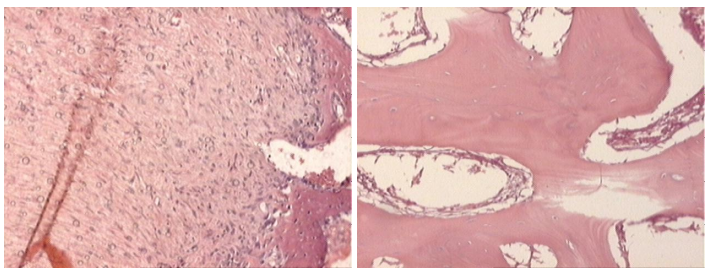

FIGURE 7 – Control x PRP. Period of 56 days (100x)

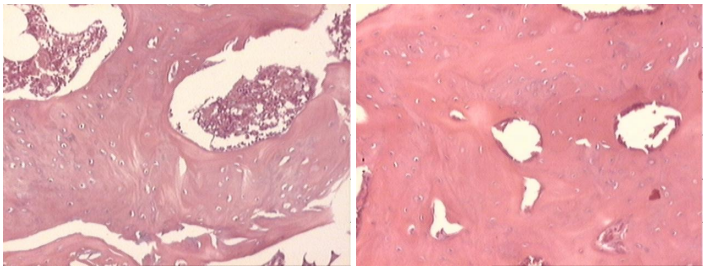

FIGURE 8 – Control x PRP. Period of 84 days (100x)

Discussion

Radiotherapy represents an established therapeutic resource for the treatment of the head and neck cancer. Approximately half of the patients with cancer use radiotherapy in some phase of the treatment of the disease, isolated or associated to other forms of therapy. However, its action is not limited to the tumoral cells, in other words, normal tissues like the epithelium, the mucous membrane, the glandular tissue and the bone which are in the radiated field are also affected by radiation.

The effects of the radiotherapy in bone were first noticed in 1922. The radiation has combined effects on the cellularity and vascularization of the bone, with reduction of the number of cells in the bone marrow, straightening of the vascular lumen, besides the increase of empty gaps in the cortical bone[1,3,8]. Soon after the radiation, the osteoblastic activity is abolished, but the osteoclastic activity keeps on and can eventually increase for a short period of time. This imbalance alters the process of bone remodeling, represented for incomplete osteons in a proportion three times bigger than the normal, and result in the increase of the porosity of the bone[1,8]. There are still structural changes in the inorganic bone content and alteration in the organic content of the bone matrix, mostly in the type I collagen[9]. The endothelial cells are also affected and the vascular fibrosis reduces the vascularization, affecting the vitality of bone and marrow cells, and making the area sensitive to infection and necrosis[10]. These effects can be noticed soon after the radiation, but the risk of late side effects is constant[2]. So, subsequent attempts of bone regeneration are variable and below normal.

The effects are radiation dose dependent, and the damage observed in the bone cells can persist for several weeks after a single application. The tissue injuries also depend on the size and volume of the radiated field, on the number and interval between sessions, on the fragmentation of the dose, on the type of radiation and on the surgical or traumatic damage to the radiated tissue. For establishment of the necessary dose in the radiotherapy, the tumoral mass must be considered. Microscopic tumors usually apply for doses between 50 and 55Gy, while doses between 60 and 70 Gy are employed in larger tumors or in advanced stages of the disease with high risk of local or regional recurrence[11]. In the bone tissue the chance of recuperation of the structure reduces considerably when the doses are greater than 60Gy, increasing osteoradionecrosis incidence; severe cases generally are associated to doses bigger than 70Gy[3,8].

In animal model, Ohrnell *et al.*[6] also found that the capacity of bone regeneration was proportionally reduced with larger doses of radiation up to the limit of 30Gy. Aitasalo[8] observed in the bone marrow of tibiae of rabbit that a dose so small as 5Gy can provoke vascular alterations the next day of the application; the destruction of osteoblasts was taking place one day after radiation with 10Gy and of the osteoclasts with 20Gy; with a dose of 30Gy, there can be persistence of edema and fibrosis. In the rat femur bone defects of 1,2 mm of diameter, Arnold *et al.*[7] noticed that a dose of 13Gy caused damage of the bone regeneration by reducing the cellular population below a critical level, while doses greater than 15Gy provoked destruction of the clot and inhibition of the regeneration process by affecting the cellular migration of regions more distant of the bone marrow.

The pathogenesis of alterations in bone tissue is related to the generation of free radicals by radiation and to the consequent damage to the endothelial cells in the field of radiation. The cellular death and the irreparable damages to the cells can cause a chronic inflammation and progressive endothelial obliteration, which results in ischemia and fibrosis in the tumoral tissue as well as in the healthy tissues. The larger effects are seen in the DNA, at the moment of major activity of the cycle of cellular division; so tissues with bigger taxes of proliferation are most affected by the radiation[7,11]. So the cumulative effect of the radiation conducts to hypovascularization of the region, tissue hypoxia, destruction of the cells of the bone tissue and fibrosis of the bone marrow[10].

At biochemical levels it is possible that hypovascularization, hypoxia and hypocellularity caused by the radiation can in part be attributed to the reduction of growth factors implicated in the tissue regeneration, like the PGDF and TGF-beta[12]. So, the use of the PRP may allow the enlargement of the concentrations of PDGF and TGF-beta, in the intention of increase the rate and quantity of bone neoformation. The growth factors are released from degranulation of the platelets, due to the contact with the collagen exposed in the wound. The principal growth factor released by the platelet granules, the PDGF reaches the cells target, binding to the receptors of the membrane and establishes connections of a tyrosin-kinase protein. It stimulates the mitogenesis of osteoblasts and of steam-cells in the bone marrow, the angiogenesis that guarantees the nutrition and the local oxygenation, the activation of macrophages, besides promoting perivascular migration of cells and modulating the effects of other growth factors. The TGF-beta is a family of local mediators and its principal activities are chemotaxia and mitogenesis of the osteoblasts precursors, the maturing of the osteoblasts, besides stimulating the deposition of collagen matrix. When TGF-beta influence the osteoclasts it inhibit bone resorption. Also they activate fibroblasts, endothelial cells, osteoprogenitor cells, condroprogenitor cells and mesenchymal cells.

The fact that PRP is an antilogous preparation introduced at the moment of the surgery also reduces the risk for transmission of diseases or immunological reactions which are associated to other materials of allogenic or xenogenic origin; besides it constitutes a material of easy attainment, low cost and simple clinical application[5].

Earlier studies have already demonstrated that cells are able to respond to the growth factors contained in the PRP[5] but more investigations are still necessary to determine the ideal concentration of the different growth factors, but the advantages of the application of the PRP in bone regeneration are undeniable. The PRP has great mitogenic effect, but its action in bone

regeneration is variable and dependent of the concentration, which was demonstrated in experimental studies[13].

In the models of bone regeneration without the influence of radiation, it seems that PRP promoted an increase of the number of osteoblasts in the initial periods of regeneration, and bone increased density of 15% to 30%[4]. In bone defects filled with autogenous bone or another osteoconductive material, the results with PRP also showed greater bone neoformation in the initial period[14]. Promising results were also obtained in studies undertaken to increase of the alveolar ridge[15]. In studies of maxilo-mandibular reconstruction with plates, autogenous bone and PRP, the result can be more obvious after a longer period of time[16].

The use of the PRP in this study aimed to accelerate the regenerative process of tissues promoting the primary stabilization and the revascularization of the bone tissue. The results found in this study suggest that the PRP had a positive effect in the regeneration of irradiated bones. The analysis of Table 1 reveals that in the control group the 21 days bone area was only superior to the 7 days period, which suggests a later and limited answer of the bone filled out only with the blood clot. This observation is in agreement with the results of Fennis *et al.*[17] in which the bone tissue irradiated with high doses became not viable for clinical purposes.

On the other side, the PRP group demonstrated a quicker and prolonged bone neoformation answer, in 14 days. The observation is ratified by the observation of Table 1, in which PRP group presented bigger bone neoformation than the control group in all periods after 7 days. Besides, mean values of bone area of the control group and PRP group are quite different. PRP group presents values on average twice superior to the control group, and in some cases up to ten times greater, as in the period of 14 days. Similarly, Aghaloo *et al.*[12] observed an increase in the expression of growth factors in 1 and 2 months after the graft of autogenous bone with PRP. The findings of this investigation also corroborate Cenni's *et al.*[18] affirmations that the bone regeneration might be favored by a better vascularization, due to the effect of PRP in the proliferation of the endothelial cells, and from Fréchette *et al.*[13], for whom it is clear that the stimulation of the formation of blood vessels increases the osteogenic activity because the angiogenesis precedes the osteogenesis and the osteblasts are perivascular cells.

The absence of differences in the first 7 days of the study does not mean that the PRP was not acting. The presence of greater bone formation after 14 days suggests that in the first days the PRP should have contributed to the formation of collagen matrix which was later mineralized and quantified in this work. Under this vision, the PRP had an effect proliferative in osteoblasts. So, the absence of difference between the groups in the initial period can be attributed to the imposed method of quantification of bone tissue in the analyzed specimens, in other words, visual identification of mineralized tissue.

In this study the PRP accelerated the bone neoformation, showing advantages in its application for the rehabilitation treatment of the irradiated bone. At the microscopic level, the PRP allowed that the hypovascularization, hypoxia and hypocellularity caused by the radiation were reduced by adding to the surgical bed the necessary growth factors for the tissue regeneration, the PGDF and TGF-beta. In clinical terms, the PRP can contribute to a regeneration of the irradiated bone of better quality, supplying growth factors that might increase the vascularization of the bone and reduce the risks of osteoradionecrosis.

Conclusion

The addition of platelet-rich plasma had benefic effect in initial cellular regeneration periods and enhanced the bone formation in later periods when compared to control.

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