7 - ORIGINAL ARTICLE
Models, Biological

Effects of a highly concentrated platelet-rich plasma on the bone repair using non-critical defects in the calvaria of rabbits[1]

Efeitos do plasma rico em plaquetas altamente concentrado no reparo ósseo, utilizando defeitos não-críticos na calvária de coelhos

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ABSTRACT

Purpose: To verify the effect of highly concentrated platelet-rich plasma (hPRP) in the pathways of bone repair using non-critical defects in the calvaria of rabbits. **Methods:** The hPRP was produced from collected venous blood of 21 rabbits. Four non-critical defects of 8 mm in diameter were created on the calvaria of each animal. The defects were all treated differently: autogenous particled bone (APB, group 1), autogenous particled bone associated with hPRP (APB + hPRP, group 2), isolated hPRP (group 3), and blood clot (control, group 4). Animals were submitted to euthanasia on the 2nd, 4th and 6th week postoperatively. Histological and histomorphometric analysis were carried through. **Results:** After two weeks, groups 1 and 2 were in more advanced stage of repair than 3 and 4. At this period, comparing the groups 1 and 2, no significant differences were found between both, which also happened between the groups 3 and 4. However, after four and six weeks, the group 1 showed a more advanced stage of repair among all the other studied groups, while group 2 was in more advanced signs of bone repair than groups 3 and 4. Comparing groups 3 and 4, after four and six weeks, the least advanced stage of bone repair was found to be within group 3. **Conclusion:** The use of a highly concentrated PRP was considered prejudicial to the repair of non-critical defects in the calvaria of rabbits, either in the association of autogenous particled bone, when compared to autogenous particled bone alone, or in its isolated form, when compared to blood clot (control).

Key words: BoneTransplantation. Bone Regeneration. Platelet-Rich, Plasma. Skull. Rabbits.

RESUMO

Objetivo: Verificar os efeitos do plasma rico em plaquetas altamente concentrado (hPRP) sobre o reparo ósseo, utilizando defeitos não críticos na calvária de coelhos. **Métodos:** O concentrado de plaquetas foi produzido a partir de sangue venoso coletado de 21 coelhos. Quatro defeitos não críticos de 8 mm de diâmetro foram criados na calvária de cada animal. Os defeitos foram tratados de modo distinto: osso autógeno particulado (grupo 1)**,** osso autógeno particulado associado com hPRP (grupo 2), hPRP de modo isolado (grupo 3) e coágulo sangüineo (controle, grupo 4). Os animais foram mortos na 2º, 4º e 6º semanas do pós-operatório. Análises histológicas e histomorfométricas foram realizadas. **Resultados:** Em duas semanas, os grupos 1 e 2 estavam num estado de reparação mais adiantado que os grupos 3 e 4. Neste período, quando comparados os grupos 1 e 2, não foram observadas diferenças estatísticamente significativas, o mesmo acontecendo quando a comparação foi entre os grupos 3 e 4. Após quatro e seis semanas, contudo, o grupo1 mostrou um estágio mais avançado de reparo, isto quando comparado com todos os outros grupos estudados, enquanto o grupo 2 apresentou sinais mais avançados de reparo que os grupos 3 e 4. Comparando os grupos 3 e 4, após 4 e 6 semanas, um estágio menos avançado do reparo ósseo foi observado no grupo 3. **Conclusão:** O uso do plasma rico em plaquetas altamente concentrado foi considerado prejudicial ao reparo de defeitos não críticos na calvária de coelhos, tanto quando em associação com enxerto ósseo autógeno em partículas (quando comparado com enxerto ósseo em partículas de forma isolada) quanto em sua forma isolada (quando comparado com o coágulo sangüíneo-controle).

Descritores: Transplante Ósseo. Regeneração Óssea. Plasma Rico em Plaquetas. Crânio. Coelhos.

[1] Research performed at the Principles of Surgery Post-Graduate Program, Evangelical Medical School, Curitiba-PR, Brazil.

Introduction

Multiple uncertainties still exist about the action of the growth factors and the platelet-rich plasma and its effect on the tissue repair. It is known that platelets are a source of several growth factors, among which are; PDGF[1-4], TGF-b[5,6], VEGF[7,8], IGF-I[4,9] and EGF[6,10]. 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[4,7,9,12], and also to improve soft tissue repair[4,10].

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[13-15].

Some studies show positive PRP results[2,7] while others present poor PRP results[5]. 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[10]. Thus, more studies in vivo and in vitro may contribute to clarify aspects of PRP use and its effectiveness.

The present study aims to analyse the effects of a highly concentrated PRP (hPRP) on the mechanism of tissue repair of non-crictical defects in the calvaria of rabbits.

Methods

Animal model and PRP preparation

This study was performed at the Institute of Medical Research (IPEM) of the Evangelic University Hospital of Curitiba, according to its Committee of Ethics. All animals used in the designed study were from the IPEM laboratory. Twenty one white rabbits (New Zealand), female, age between 350-370 days, weight of 2,750-4,600g, with no previous disease, were used.

Animals were anesthetized with an intramuscular injection of ketamine 5% (0,5mL/Kg). For the collection of venous blood the most favorable ear vein of each animal was punctioned, using a scalp 21. Afterwards, a seringe of 20 mL with 10% sodium citrate was connected to the scalp.

Approximately 15 mL of blood of each rabbit was collected; 10 mL were transferred to a tube 16 x 100 mm and 4 mL to other tube 12 x 75 mm. Automatic platelet counting was done using the blood of the 12 x 75 mm tube.

For the preparation of the hPRP a regular centriphuge was used (Excelsa Baby II 206-R). At first, tubes were placed on 200g for 20 minutes, allowing the formation of two distinct fractions; plasma at the superior part of the tube (slightly yellow colored) and the blood cells fraction at the bottom (red colored). All of the plasma fraction plus the upper (1 mL) part of the blood cells fraction were transferred to another tube, submitted to a second cycle of 400g for 10 minutes. After this last cycle, two distinct fractions could be identified. The upper fraction (yellow colored) was removed to the point where its remanescent plus the bottom fraction (red colored) completed a total of 1 mL. After homogeneization, 1 mL of final product - from the initial 10 mL of blood was obtained. Another automatic counting of platelet was done.

Surgical procedure

The region was previously shaved and asseptically prepared. Sterile barriers limited surgical field. The region was injected sub-periosteally with 1 mL of lidocaine 2% with adrenaline 1:100.000. A midline dermo-periosteal incision (5 cm) was made, raising a skin-periosteal flap to expose the calvarial surface. Four defects of 8 mm in diameter were created with a trephine under profuse saline solution irrigation. Bone fragments were particled for grafting. For the hPRP coagulation, a mixture of 10% calcium chloride solution and 5000 units of bovine thrombin were added to the previously prepared hPRP. (1 minute for gel obtaintion). One defect was grafted with autogenous particled bone (APB, group 1), another with autogenous particled bone associated with hPRP (APB + hPRP, group 2), isolated hPRP (group 3), and no grafting (control, group 4) (Figure 1, Table 1). Tissue flaps of the wound were ininterrupted sutured.

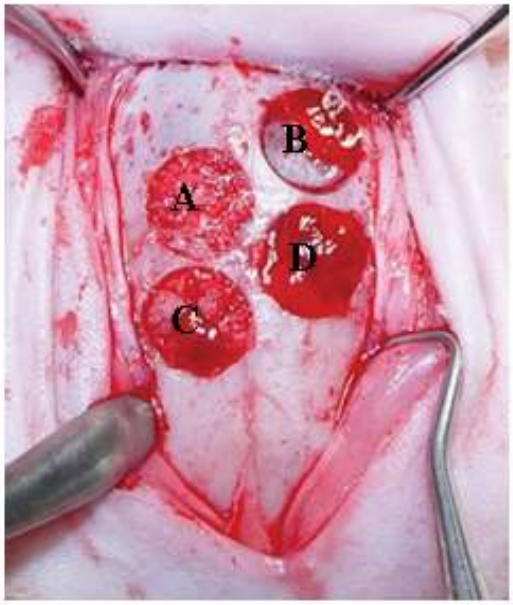

FIGURE 1 - Bone defects grafted with autogenous particled bone (APB) (**A**), APB associated with highly concentrated platelet-rich plasma (APB + hPRP) (**B**), isolated hPRP (**C**), and no grafting or control (**D**)

TABLE 1 – Animal groups according to type and time of the experiment.
Study design

Animals	Monitoring period (weeks)	Groups	Treatment
1-7	2	1	APB
		2	APB + hPRP
		3	hPRP
		4	Control
1-7	4	1	APB
		2	APB + hPRP
		3	hPRP
		4	Control
1-7	6	1	APB
		2	APB + hPRP
		3	hPRP
		4	Control

APB = autogenous particled bone; hPRP = highly concentrated platelet-rich plasma.

Histologic and histomorphometric assessments

The animals were dead with an overdose of the anaesthetic solution after two weeks (7 animals), 4 weeks (7 animals), and 6 weeks (7 animals). Block specimens were obtained using an inverted cone bur. Sections of 5µm were obtained and stained with Giemnsa.

Image acquiring was done with the use of a light microscope (21/3, Quimis) and camera (SDC-310) according to a previously published methodology[16]. Three randomly selected microscopic fields within each grafted area from all groups and animals were analysed.

Histomorphometric parameters were analysed using the UTHSCSA Image Tool 2.00. Areas (mm^2) of grafted bone in relation to total area (two weeks only), areas of mature bone, immature bone, osteoyd (with osteoblastic profliferation), medullar regions (four and six weeks only) and granulation tissue were measured (Figure 2). A total of approximattely 2 mm^2 were analysed in each field. Data were recorded in mm^2 and percentage for each parameter.

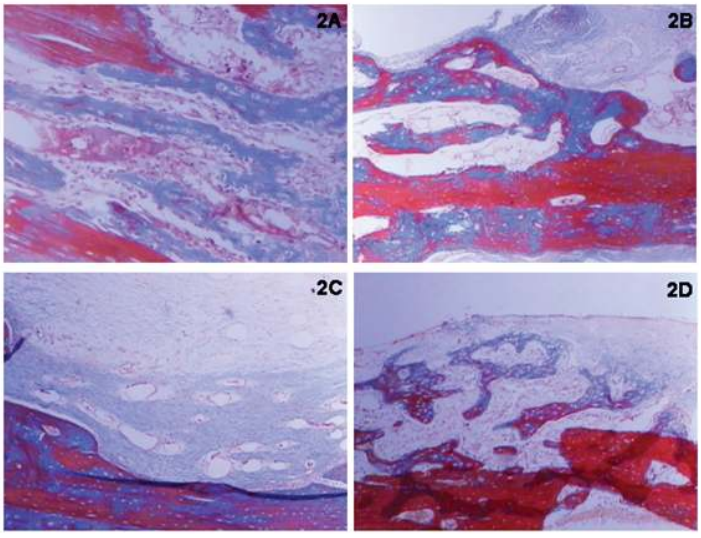

FIGURE 2 - Fragments of mature bone (probably from grafting) and granulation tissue with intense cellularity, autogenous bone (**2A**). Granulation tissue mildly vascularized with intense osteoblastic differentiation, autogenous bone associated with hPRP (**2B**). Dense connective tissue compatible to granulation tissue, hPRP only (**2C**). Connective tissue within a trabecular bone matrix in maturation stage, control (**2D**). (Masson trichrome - magnification 40x)

Statistical analysis

Within each monitoring period (weeks), Friedman's nonparametric "t" test was done. In the case of significant difference among treatments, the Wilcoxon's nonparanetric test was done. Values of $p<0.05$ indicated statistical significance.

Results

Platelets counting analysis

Medium values, minimum and maximum values were obtained for all of the countings (Table 2).

TABLE 2 - Minimum, medium and maximum value with standart deviation (s.d.) of variables

Variable	Minimum Value	Medium Value	Maximum Value	Mean ± s.d.
Weight (g)	2,750	3,500	4,400	3,572 ± 448
Initial counting of platelets	103,000	309,000	500,000	301,400 ± 105,632
hPRP counting of platelets	612,000	2,150,000	6,880,000	2,414,720 ± 1,547,862
Enrichment (%)	236	595	1,759	687 ± 380

The used method for the production of the highly concentrated PRP in this study allowed for a mean enrichment of 687% of platelets, wich means that the hPRP counting of platelets was approximately seven times higher than initial counting of platelets.

Morphological microscopic analysis

After two weeks, group 1 (APB) presented in the repair of bone defect a loose connective tissue mildly vascularized, with low cellularity, either of fusiform-like (similar to mature fibroblast) or of star-like aspect (similar to immature or young fibroblast, or even at an osteoblastic differentiation stage). It can also be observed, at the center of the defect, fragments of mature bone (probably from grafting) with osteoclastic activity. In the surrounding areas of the defect a neoformation of immature bone tissue was observed, with intense osteoblastic activity. The group 2 (APB + hPRP) presented a granulation tissue mildly vascularized in all cases, with intense cellularity, either of fusiform-like or star-like aspect, similar to young or immature fibroblast or in osteoblastic differentiation. Also, fragments of mature bone tissue (grafted origin) with osteoblastic and osteoclastic activity were noticed. In the surrounding areas, an immature bone neoformation with intense osteoblastic activity occurred. The group 3 (hPRP) presented a loose and dense connective tissue compatible to granulation tissue, with fusiform-like and osteoblastic cells. Also, intense and diffuse inflammatory process, especially eosinofilic, was observed within the defect. In the surrounding areas, intense osteoblastic activity with osteoyd tissue was seen. The group 4 (control) presented intense angiogenic area with dilated blood vessels. Within this area a trabecullar bone matrix in maturation stage was also found (Figure 2).

After four weeks the group 1 (APB) showed intense osteoblastic activity with hyalinized stroma similar to osteoyd. The group 2 (APB + hPRP) showed either loose or dense connective tissue, compatible with granulation tissue, similar to what was observed in two weeks, nevertheless more bone tissue was noticed

peripherically. The group 3 (hPRP) showed similar histological aspects to two weeks, but without inflammatory process. Continue and immature bone trabeculae were observed within granulation tissue in the medullar region of defect. While in the group 4 (control) the aspect was also similar to two weeks, with mature bone trabeculae at central regions and immature bone peripherically, in a highly vascularized stroma (Figure 3).

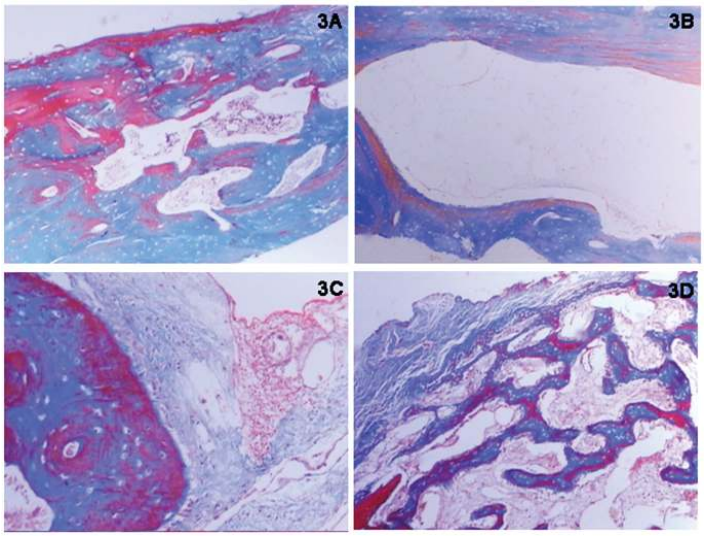

FIGURE 3 - Bone maturation and medullary structure with intense osteoblastic activity, autogenous bone (**3A**). Large trabeculae within medullary structure, autogenous bone associated with hPRP (**3B**). Granulation tissue presence, hPRP only (**3C**). Highly vascularized stroma with centrally mature bone and peripherically immature bone of trabeculae, control (**3D**). (Masson trichrome - magnification 40x)

After six weeks the group 1 (APB) demonstrated bone trabeculae in highly vascularized stroma. The group 2 (APB + hPRP) demonstrated bone tissue fragment similar to medullar aspect, likewise group 1 (APB), but with bone trabeculae of smaller widths. The group 3 (hPRP) demonstrated loose connective tissue highly vascularized with several fragments of bone tissue, with no trabeculae organization present yet. And finally, the group 4 (control) demonstrated a similar aspect to group 1, although with more sparse trabeculae layers (APB) (Figure 4).

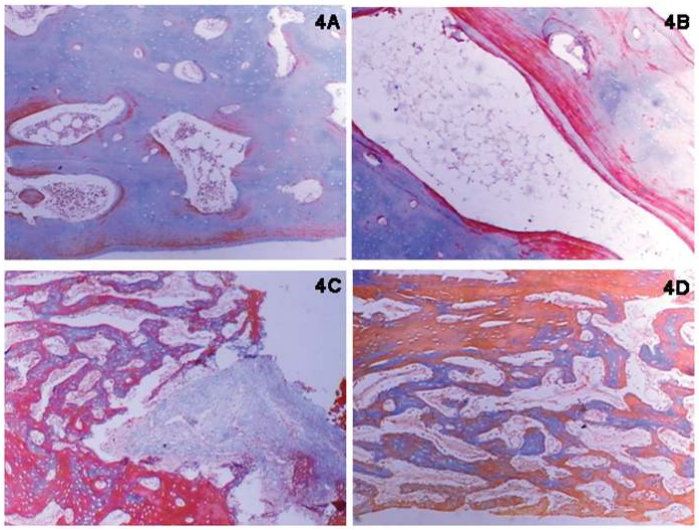

FIGURE 4 - Trabecular mature bone, autogenous bone (**4A**). Large trabeculae within medullary structure, autogenous bone associated with hPRP (**4B**). Granulation tissue presence among immature bone, hPRP only (**4C**). Granulation tissue within mature and immature bone, control (**4D**). (Masson trichrome - magnification 40x)

Histomorphometric analysis

After two weeks, groups 1 (APB) and 2 (APB + hPRP) demonstrated means of 31.42% and 26.29%, respectively, of mature bone (MB) in relation to the total area of the defect. This mature bone found at this period demonstrated two degrees of bone; one considered grafted mature bone (GMB) and other neoformated mature bone (NMB). For these groups, the percentages of GMB were 23.87% and 19.25%, for groups 1 and 2 respectively; while the percentages of NMB were 7.60% and 7.03%, respectively. Also, the amounts of immature bone seen in both groups were 8.47% and 5.81%, for group 1 and 2. Comparing the groups 3 (hPRP) and 4 (control), group 4 presented a higher percentage of mature bone (3 = 7.10%; 4 = 9.40%); for immature bone the results showed 18.50% (group 3) and 20.93% (group 4) – not stastiscally significant.

After 4 weeks, the percentage of mature bone in group 2 (35.99%) was higher than in groups 3 (16.15%) and 4 (19.07%) – results statistically significant. In group 1, the presence of mature bone was 54.18%, significantly higher than all the other studied groups. Comparing the amounts of immature bone, group 4 showed the highest percentage (4 = 42.02%; 3 = 17.34%; 1 = 16.01%; 2 = 14.94%).

After 6 weeks, group 1 continued showing the highest percentage of mature bone when compared with all others (1 = 52.06%; 2 = 36.55%; 4 = 28.06%; 3 = 22.16%). Comparing the presence of immature bone, group 4 (control) showed the highest percentage (4 = 25.61%; 3 = 12.44%; 1 = 12.30%; 2 = 9.29%).

In relationship to the presence of granulation tissue, the groups with hPRP (groups 2 and 3) presented the highest percentages at four and six weeks; at four weeks, the amounts of granulation tissue were 3 = 31.00%, 2 = 25.91%, 1 = 7.70% and 4 = 6.83%; at six weeks the amounts were 3 = 20.28%, 2 = 8.46%, 1 = 2.41% and 4 = 1.94%.

Discussion

It is known that platelets are a source of several growth factors. The PDGF and the TGF-β seem to be the main growth factors in the PRP, once they are always mentioned[1,2,3,7,9,12]. Many doubts still remain regarding the effectiveness of the PRP and the growth factors on the mechanism of tissue repair.

In humans, many authors demonstrate positive results with the use of PRP in situations of tissue repair[2,3,17,18-22], while others, however, do not observe benefits[9,23].

Sanchez *et al.*[24] affirm that there is a lack of scientific evidences to support the use of PRP in combination with graftings in reconstructive procedures, and Frymiller and Aghaloo[8] establish doubts if PRP can be recommended for the use in humans.

In animals, several studies had been developed with application of PRP. Some of these studies also use rabbits[5,25,26]. Aghaloo *et al.*[5] show a very similar study to this presented here, with differences in the preparation of the PRP, in the monitoring periods (two, four and six weeks here and one, two and four months in theirs) and in the results when in the current study the PRP seemed to be harmful to the repair, these authors practically demonstrate neutral action of the PRP.

Dogs also were used in studies with PRP. Kim *et al.*[25] demonstrate positive results in the association of implants, dried frozen bovine bone and PRP. Suba *et al.*[27] associate the beta-

tricalcium phosphate to the PRP with positive results, even at initial phases of repair. Gerard *et al*.[28] also demonstrate favorable results at initial periods of repair with the association of cortico-medular autogenous bone and PRP. Poor results are demonstrated by Choi *et al*.[15], and for Jensen *et al*.[29].

Sheep were used by Jakse *et al*.[30] (autogenous bone and PRP) and by Sarkar *et al*.[31] (collagen sponge and PRP) - in both the studies the results with PRP were not advantageous. Fürst *et al*.[32], Wiltfang *et al*.[18] and Klongnoi *et al*.[33] use minipigs as experimental animal. Fürst *et al*.[32] (bovine bone) and Klongnoi *et al*.[33] (autogenous bone, bioactive glass) do not demonstrate positive results with PRP. However, Wiltfang *et al*.[18] (autogenous bone) show positive results with the use of the PRP.

In goats, autogenous bone showed considerable improvements in the bone repair, with increase of the revascularization, reduction of the reabsortion and increase of the bone neoformation, mainly after six and 12 weeks; the beneficial effect of the PRP were also observed later on[19]. Although it is known that the growth factors are delivered during few days after the local application, according to these authors, their initial effect can be important once they can promote the revascularization and also tissue acceptance of the grafted material. In the current study, the opposite was observed; perhaps an initial problem with the revascularization or acceptance of the grafted material may be the key factor for the impaired results observed later.

In rats, Fontana *et al*.[34] (titanium laminar implant), Pryor *et al*.[35] (collagen sponge) and Plachokova *et al*.[36] (beta-tricalcium phosphate, hidroxiapatite) did not find results that justify the use of PRP. However, Weibrich *et al*.[14] (implants) while studying different levels of PRP concentration, found positive results with concentrations lower than six times, and observed that the use of highly concentrated PRP (6-11 times) inhibits the bone repair. The technique used for the production of the PRP in the current study was capable to produce mean platelets concentrations higher than six times the concentrations verified in the peripheral venous blood. Perhaps this high concentration has contributed for the poor results in this study.

Studies in vitro supply with interesting data regarding the PRP. Arpormaeklong *et al*.[13] (rat bone marrow stromal cells, rhBMP2, PPP = platelet-poor plasma) demonstrated the PRP as estimulator of the cellular proliferation (dose-dependent), and inhibitor of the activity of alkaline phosphatase and calcium deposition. Choi *et al*.[37] (alveolar bone cells, PPP, PC = platelets concentrate without plasma) observed that PRP in high concentrations suppress the viability and the cellular proliferation, however there is a stimulation effect in low concentrations (1-5%). Also observed cytotoxic answers with the PPP, and with PC an increase in the viability and the proliferation cellular of significant form. Such results raise the question about the plasma itself as responsible for the negative effects found; likewise the results observed in the current study, which could possibly be attributed to the amount of plasma in PRP.

Annunziata *et al*.[38] report that PRP stimulates the growth of periodontal ligament human cells (dose-dependent) and the proliferation of gingival fibroblasts, also inhibits the growth of human keratinocytes and increases the activity of alkaline phosphatase. Biological effect of the PRP on the proliferation and differentiation of human osteoblast-like cells was already observed, increasing cell viability (dose-dependent) and suppressing the activity of alkaline phosphatase in the phase of cellular growth, although enhancing the enzyme action when cells reach confluence.

Graziani *et al*.[6] studied the effect of different concentrations of PRP on human osteoblasts and fibroblasts. Maximum stimulation effects are found in the concentrations of 2.5x. Higher concentrations of PRP increase the amounts of TGF-b 1 and osteocalcin, and diminish the osteoprotegerin levels, stimulating osteoclastogenesis and osteoblastic differentiation; a nocive effect once during the initial phases of the repair it is essential that the proliferation occurs before the differentiation. These results also are in agreement with the non-favorable results found in the use of the highly concentrated PRP demonstrated in the current study.

Conclusion

The use of a highly concentrated PRP was considered prejudicial to the repair of non-critical defects in the calvaria of rabbits, either in the association of autogenous particled bone, when compared to autogenous particled bone alone, or in its isolated form, when compared to blood clot (control).

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