Effect of bone marrow mononuclear cells plus platelet-rich plasma in femoral bone repair model in rats

Modelo experimental em ratos no reparo ósseo do fêmur utilizando células mononucleares no plasma rico em plaquetas

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Abstract

Mononuclear cells from bone marrow have been used in various treatments of diseases in an attempt to regenerate tissues. The objective of this study was to evaluate the accession and proliferation of mononuclear cells in a critical defect, with the addition of platelet-rich plasma (PRP) and / or TGF-β1 (transforming growth factor beta 1), and then to evaluate bone repair at defective sites on the femurs of rats. We created a critical defect on the bilateral femurs of 33 Wistar-Kyoto rats. Mononuclear bone marrow cells, TGF-β and PRP were added to the lesion on the treated side and saline on the contralateral side. We determined the adhesion of mononuclear cells at the critical defect and bone repair. The presence and consequent adhesion of the mononuclear cells administered to the treated animals was not demonstrated by PCR. Radiographic analysis showed closure of the lesion, but we could not affirm that this resulted from the treatments administered or by normal bone regeneration itself, when the lesion was evaluated at 6 and 10 weeks postoperative, seeing that there were no significant differences between the groups. Conclusions: a) Bone marrow mononuclear cells did not adhere at the critical defect created in the rat femur; b) It was not possible to determine the efficiency of the bone repair treatments studied, as the results did not show significant differences between the groups.

Keywords: Critical defect. Femur. PRP. TGF-β1. Rats.

Resumo

As células mononucleares da medula óssea têm sido utilizadas em diversas afecções na tentativa de regeneração tecidual. O objetivo deste estudo foi o de avaliar a adesão das células mononucleares sobre defeito crítico, com a adição do plasma rico em plaquetas PRP) e / ou TGF-β1 (Fator de crescimento transformador Beta 1), e, por fim, verificar o reparo ósseo dos sítios defeituosos dos fêmures dos ratos. Foi criado um defeito bilateral critico nos fêmures de 33 Wistar-Kyoto ratos. Células mononucleares de medula óssea, TGF-β e PRP foram adicionadas no lado tratado da lesão e soro fisiológico no lado contralateral. Determinou-se a adesão de células mononucleares sobre o defeito crítico e reparo ósseo. A presença e consequente adesão das células mononucleares administradas nos animais tratados não foi evidenciada através da técnica de PCR. As análises radiológicas evidenciam fechamento da lesão, porém, nós não podemos afirmar que foi pelos tratamentos administrados ou pela própria regeneração óssea, quando analisadas em seis e 10 semanas pós-operatórias, haja visto não apresentarem diferenças significantes entre os grupos. Conclusões: a) As células mononucleares não aderiram ao defeito crítico criado no fêmur do rato; b) Não foi possível avaliar a eficiência dos tratamentos propostos para o reparo ósseo, por não apresentarem diferenças significativas entre os grupos.

Palavras-chaves: Defeito crítico. Fêmur. PRP. TGF-β1.Ratos.

Introduction

The possibility of the regeneration of tissues and organs has been the subject of research over the years. Thus, new areas of medicine are investigating techniques for tissue regeneration from natural regulators (growth factors) and components of the organism itself (stem cells)[1].

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Two approaches of particular interest to researches are pursued for the advancement of the scientific basis of clinical application, creating new parameters for the production of tissues and the development of procedures for tissue repair and regeneration in vivo, as well[2].

Bone tissue is constantly in a dynamic morphological process following its original formation. Thus, bone metabolism is maintained by a constant process of bone resorption and formation in response to physical and biomechanical changes. This event is controlled by two mechanisms: local and systemic regulation, through hormones that control the levels of calcium and phosphate[3,4,5]. Platelet-rich plasma (PRP) is a product of the laboratory processing of autologous blood, and its therapeutic strategy is to accelerate wound healing through the concentration of growth factors present in platelet cytoplasm, which are universal indicators of almost all wound healing events[6] and free from toxic characteristics or immunoreactivity[3].

Studies described in the literature have shown that in the fraction of mononuclear cells obtained from bone marrow, umbilical cord and other tissues, there are both hematopoietic stem cells and mesenchymal stem cells[7,8]. The hematopoietic stem cells give rise to all the different cells of the blood, and are characterized by the membrane protein CD34. The mesenchymal stem cells give rise to cells that will form the other tissues of the body, and they express the membrane markers CD105 (endoglin) and CD117 (c-kit)[9,10,11]. Thus, it is possible to select the specific cell populations most suitable for the regeneration of the tissue of interest.

Material and Method

We used for this study 39 outbred Wistar-Kyoto rats, weighing between 200 and 250 g. Six male rats were used as bone marrow donors and 33 females rats, used for the intervention protocol. The rats were obtained from the animal facility of PUCRS; they were housed in individual cages and received water and food ad libitum. The rats were kept on a 12-h light/dark biological cycle.

Description of growth factors used in the protocol and their origin

a) TGF-β1 - Transforming growth factor beta 1 in the protocol. The product was purchased from Biosource Laboratory and had a purity of >98%. It was reconstituted with sterile distilled water to a concentration of 50 mg/mL. We used a concentration of 6 ng/mL of TGF-β1 in a volume of 30 μL adapted so that there was no leakage of fluid in the critical defect created[12].

b) Isolation of PRP (platelet-rich plasma) from the carotid artery of rats via cardiac puncture.

PRP was isolated by cardiac puncture, according to the protocol of Plachokova et al.[13] and the PRP fraction was purified as described by Rossi Junior, Leme and Pispico[14]. We used 30 μL of PRP in the critical defect created[15]. A hemogram was performed in order to determine the number of platelets present in the PRP samples from 3 rats used for the treatment procedures. An automated Coulter STKS counter, at Hospital São Lucas from PUCRS, was used according to the manufacturer's instructions. A 150 uL aliquot of each PRP sample was counted and showed the following results: animal group 1 - platelets in PRP = 1,650,000 (1,650 103/uL); animal group 2 - platelets in PRP = 2,247,000 (2,247 103/uL); animal group 3 - platelets in PRP = 2,280,000 (2,280 103/uL).

c) Isolation of mononuclear cells from rat bone marrow.

MCs were isolated from the bone marrow donor (1 male rat for each study group) after sacrificing the animal with intracardiac overdose of thiopental (2 mL, equivalent to 100mg /kg rat). The mononuclear fraction of bone marrow was obtained by flushing the femurs, tibias, humeri and ulnae of animals. We collected the bones, and after they were washed with DPBS solution (Gibco®, USA – Invitrogen- São

Paulo, SP, Brazil), bone epiphyses were cut. The extraction of bone marrow stroma was obtained by the introduction of a 32 G needle in the bone ends, and the cavities later washed with heparinized DPBS. The content was centrifuged at 1500 rpm and resuspended in RPMI 1640 (1:1) (Gibco®, USA- Invitrogen-São Paulo, SP, Brazil). This suspension was fractionated in a density gradient centrifugation using 1.191 g/L Histopaque ® (Sigma-Aldrich ®, Saint Louis, Missouri, USA) at 400 g for 30 min at 25 °C. The mononuclear fraction located at the interface with Histopaque® was collected and washed twice with saline.

Protocol establishing the critical defect

Anesthesia was induced with the animal under restraint and in the supine position. Anesthetics were administered intraperitoneally using an insulin needle and syringe. The rats were given ketamine hydrochloride (Ketamin®, Cristália Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil) (80 mg/kg mouse weight) combined with chlorpromazine hydrochloride (Longactil®, Cristália Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil) (2 mg/kg mouse weight)[16]. A 5 mm critical defect was created in the femurs bilaterally, following the protocol of Meinel et al.[17]. The rats used for this study were divided into three groups of 11 rats each, where the control procedure and treatment were performed in the same rat. The animals were under anesthesia, as described above, and after confirming the effect of anesthesia, the hind legs were shaved. For treatment, animals were placed on their side and a transverse incision was made, about 2 cm with a Carbon Steel 15 scalpel blade (Feather Safety Razor Co. Ltd, Osaka, Japan) on the right and left thigh. To expose the femur, the biceps femoris muscle was retracted posteriorly as the gluteus maximus, tensor fascia lata and vastus lateralis were retracted anteriorly. We used a retractor to expose the bone. Next, the periosteum was detached using a 19cm Freer (Neumar). A long carbide drill, No. 8, was connected to a high-speed Dental Beltec Mini ™ motor (NetDental, São Paulo, SP, Brazil) (maximum speed of 15,000 rpm drive) to drill the femur while the tissue was manually irrigated with saline and drained with a Nevoni Vacuum Aspirator ™ (LF Equipamentos, São Paulo, SP, Brazil). The critical defect was in the cavity of the medial diaphysis, the between the rough line and third trochanter of about 5 mm long by 2.5 mm wide and deep enough to reach the medullary canal[17] for application of mononuclear cells and the mixture of PRP/TGF-β in the right femur and saline in the left femur. Each treatment led to a study group: Group 1 - use of MC with 30 μL transforming growth factor beta (TGF-β); Group 2 - use of MC with 30 μL PRP (platelet-rich plasma); Group 3 - use of MC with 30 μL transforming growth factor beta (TGF-β) and 30 μL PRP (platelet-rich plasma).

After that, there was a simple suture with a Mayo-Hegar Videa tipped needle holder and Ethilon No. 5 mononylon suture of Ethicon ™ (ISP Dental, São Paulo, SP, Brazil). The suture was performed in compliance with the structure. First was the sternum periosteum, followed by the union of the muscle layers and, finally, the skin tissue. The same suture procedure was performed the left side. Postoperatively, in the first 24 h, we used as analgesia ketoprofen 5 mg/kg subcutaneous[18]. After 48 h, 200 mg/mL paracetamol (Laboratório de Habilidades Cirúrgica PUCRS, Porto Alegre, RS, Brazil) was used as analgesia with a dose of 1 mL/20mL of H_2O, equivalent to a dose of 125 to 150 mg paracetamol/day.

Radiographic examinations to follow bone growth in lesions

To carry out the images, we selected the mammography equipment Mamomat 3000 Siemens with FUJI CR technology. We used the image transducers Image Plate (IP) FUJI specific for mammography. The technology applied to mammography was selected given the size characteristics of the rats and contrast of the image for later analysis. An FCR Profect CS FUJI reader (model CR-IR 363) was used for image pro-

cessing. The radiographic technique used consisted of a Mo-Mo anode-filter combination at tube accelerating voltage of 28 kVp, and 14 mAs (an appropriate technique for imaging the lesion in the femur of these animals). The X-ray was taken with each rat placed in the central area of irradiation in the ventral decubitus position. After reading the IP, the images were saved in dicom format, identifying the group and the number of animals in each dataset collection, as well as weeks of treatment (the sixth or tenth). According with figures 1 and 2.

Euthanasia

The animals were sacrificed 10 weeks after surgical treatment, following the protocol of Kohn et al.[16], for later histological analysis. All procedures were approved by the Ethics Committee for Animal Use - CEUA / PUCRS.

Identification of the DNA of mononuclear cells - DNA extraction

DNA extraction was performed with phenol / chloroform based on the method described by Isola et al[19].

Molecular analysis– PCR

The cells administered to the lesions were identified using the polymerase chain reaction technique (PCR), following the protocol described by Mullis et al.[20] .

Histochemistry

For histological technique was used the picrosirius technique, which is a specific histochemical method for the detection of structures composed of oriented collagen molecules[21]. The picrosirius method causes large amounts of molecules of Sirius Red with acid character and elongated, to be placed in parallel with the collagen molecules, which causes considerable increase in birefringence of collagen fibers when observed under polarized light[22].

The femurs were decalcified with 5% formic acid in DPBS. The dehydrated material was embedded in paraffin using a Leica TP 1020 tissue processor (Laboratório PUCRS, Porto Alegre, RS, Brazil) The femurs were embedded in paraffin and 5μm sections of the lesion were made using micrometer Olympus CUT 4060 (Laboratório PUCRS, Porto Alegre, RS, Brazil). The material was placed in an oven at 60 °C overnight. Afterward, they were deparaffinized (xylene 2X for 5 min, absolute alcohol - 4X for 2 min) and hydrated in water. We then carried out the incubation with 1% solution of picrosirius staining for one hour followed by washing in running tap water for 20 min. The slides were dehydrated and mounted with Canada balsam. The images of histological sections were captured by a video camera mounted on an Olympus BX40 microscope and analyzed with the aid of the software Image Pro-Plus 6.1, as shown in figures 1 and 2, treated group and control group.

Statistical analysis

The results of the X-ray images were analyzed using ANOVA (one way). The comparison of the times was performed by Student's t test. The level of significance was $p < 0.05$ as statistical difference and statistical power of 95%. The data were analyzed using SPSS 14.0. The data analysis for both radiography and histology were entered into a spreadsheet (Microsoft Office Excel 2003).

Results

Radiographic evaluation

The radiographic evaluation in the postoperative period between 6 and 10 weeks showed different results between the three treatment groups. Group 1, MC + TGF-β, showed a reduction rate of 62% better in treated than in control. Group 2, MC + PRP, performed better with the rate of reduction being 66% greater in treatment than in control. Group 3, MC + PRP + TGF-β showed a reduction rate of 66% better in control compared to treatment, which indicates that this treatment effectively inhibits bone repair. The same behavior was observed with the length of the lesion where group 1 and 2 had on average 9.8% and 12.3% reduction, respectively, while group 3

showed 10.6% bone repair in the lesion, when intercomparing control with treated bone. The average reduction rate for the three groups was approximately 0.923%, which is not statistically significant between the control and treated bone.

Adhesion of mononuclear cells

The presence and subsequent adhesion of mononuclear cells administered to the treated animals was determined by PCR using primers complementary to the sequence of the marker gene TSPY.

Histological evaluation using picrosirius technique

The evaluation was made on a sample of animals per group at 10 weeks postoperative. The evaluation of histological slides showed new bone formation, with the presence of collagen for both the control and treated femurs. With the aid of Image-Pro Plus 6.1 (Media Cybernetics, Inc.), the area corresponding to the bone of each image was selected. In the bone area of the image, with normal bone tissue expressed as 100% neoformation (ideal) for comparison, we calculated the percentage of bone neoformation of the treated area compared to normal bone. Based on the number of specimens used in this study, the groups tested showed no statistically significant differences in the percentage of new bone formation promoted by these treatments compared to control. Histological analysis indicated that both the treated group and the control group showed collagen neoformation, but there was no difference between the treatments ($p = 0.296$).

Discussion

According to Rose and Oreffo[1], the Regenerative Medicine composes a new field that investigates tissue regeneration techniques from natural markers (growth factors) and components of the organism itself (stem cells). A femur fracture contributes considerably to morbidity and mortality, especially in older patients due to pre-existing diseases and complications resulting from containment.

The purpose of this study was to evaluate the therapeutic potential of the mononuclear fraction of bone marrow cells, as well as the effectiveness of platelet-rich plasma (PRP) and transforming growth factor (TGF-beta) and bone repair.

The animals used in this study, Wistar-Kyoto rats, were genetically identical, avoiding any risk of rejection of the cells used. In our study, the proposed treatments presented different results. Groups 1 and 2, MC + TGF-β and MC + PRP, showed a positive closure of the lesion, but there was no indication that one treatment was better than the other. After 6 weeks, a plateau is reached with a decrease in new bone formation. In group 3, MC + PRP + TGF-β, there was a better closure in the control group, i.e., there was a faster rate of reduction in tissue damage in the control group. The authors suggested that at 4 weeks the bones treated with PRP activated the TGF-β stored in bone compared to control for acceleration of osteogenesis. This supernatant can be spread around the critical defect treated with PRP inhibiting bone repair in the treated bone. This would explain why the control had a positive closure response in relation to the treated bone after 6 and 10 weeks, when analyzed by X-rays.

It is imperative, therefore, to establish conditions that can regenerate bone tissue in clinical situations considered inhospitable, as in places that have compromised blood supply in areas of difficult consolidation in regions that have defects with critical dimensions or where the tissue is completely absent.

To evaluate the formation of collagen and show new bone formation, were prepared histological slides, and images were analyzed in a specific software Image-Pro Plus 6.1. With this program, select the area of interest and the program itself made by counting the number of pixel area. The treated area is compared with the untreated area (which is defined as 100% control of bone formation), adjacent to the treated area with a length and width similar to the treatment area analyzed in the same histological slide.

Thus, the results of histological analyzes indicate that there was formation of collagen showing new bone formation, both for the treated group as control. However, do not indicate that this training may have been influenced by the treatments proposed, there has not submitted seen significant results from the proposed treatment.

Conclusions

a) Bone marrow mononuclear cells did not adhere at the critical defect created in the rat femur;

b) It was not possible to determine the efficiency of the bone repair treatments studied, as the results did not show significant differences between the groups.

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