

Clinical and radiographic characterization of xenotransplantation of rat bone marrow-derived mesenchymal stem cells for repair of radial defects of rabbit

Betânia MONTEIRO^{1*}, Lukiya FAVARATO², Pablo CARVALHO³, Barbara OKANO², Maria Antônia MENEGATTI¹,
Alvaro OLIVEIRA¹, Bianka SANTOS¹, Ricardo DEL CARLO⁴

¹Stem Cell and Cellular Therapy Laboratory, Animal Science, Vila Velha University, Vila Velha, Brazil

²Department of Veterinary Science, Federal University of Viçosa, Viçosa, Brazil

³Department of Veterinary Science, Federal University of Minas Gerais, Belo Horizonte, Brazil

⁴Department of Veterinary Science, Federal University of Viçosa, Viçosa, Brazil

Received: 22.05.2014 • Accepted/Published Online: 20.11.2014 • Printed: 30.10.2015

Abstract: This study aimed to evaluate the clinical and radiographic aspects of xenogenic transplantation of rat bone marrow-derived mesenchymal stem cells (MSCs) for the repair of radial bone defects of rabbit. Bone marrow derived MSCs collected from Wistar rats were expanded, differentiated, and labeled in the laboratory. In the fourth passage, the cultures were pelleted in aliquots of 10^7 cells/50 μ L and transplanted in situ into the lesions. Thirty-six rabbits were used in the in vivo study. In all animals, defects were created by removing a bone segment of 1.0 cm in length in the right radius. The rabbits were divided into four experimental groups: the control group (CG) received percutaneously administered 50 μ L of phosphate buffer (PBS) in the radial bone defect region 24 h after lesion creation; group 1 (G1) had the radial bone defect filled with absorbable gelatin sponge soaked with 50 μ L of PBS during the surgery; group 2 (G2) had the radial bone defect filled with absorbable gelatin sponge soaked with the MSC pellet; and group 3 (G3) received percutaneously the MSC pellet in the radial bone defect region 24 h after lesion creation. No clinical and radiographic changes consistent with cellular rejection were observed. Percutaneous MSC xenotransplantation contributed to the repair of experimental radial bone defects.

Key words: Bone repair, cell therapy, collagen, immune response, stem cell

1. Introduction

Mesenchymal stem cells (MSCs) are defined as a population of multipotent cells able to differentiate and produce any cell type needed in a repair process, such as osteoblasts, chondroblasts, neurons, epithelial cells, and cardiac cells (1,2).

This cell type has become the focus of numerous studies worldwide for providing clinically promising perspectives for cell therapy and also for its immunomodulatory potential (3,4), although the mechanisms of immunosuppression on inflammatory response and the mechanisms of transplant rejection are not fully elucidated (5).

Recent studies have described the use of allogeneic and autologous MSCs for the repair of various tissues (4,6). However, there is little research involving xenotransplantation in animals and most of them only evaluated the cellular interaction in vitro between MSCs and T lymphocytes.

Because of the great therapeutic potential of MSCs, in addition to the persistent doubts about their immunosuppressive capacity *in vivo*, further studies

are needed to investigate the real potential of xenogenic transplantation using these cells for tissue repair in animals. Therefore, this study evaluated clinical and radiographic aspects of xenogenic transplantation of rat bone marrow-derived MSCs for the repair of radial bone defects created in rabbits.

2. Materials and methods

This study was approved by the Institutional Animal Care Committee (protocol 97/2010).

2.1 Cellular culture

A total of five male, 4-week old Wistar rats were euthanized using anesthetic overdose. The animals were immersed in alcohol 70° to ensure antisepsis for cell collection and were taken to the laminar flow cabinet. The femurs were disarticulated and removed aseptically. The distal epiphyses were cut and the medullary canal was flushed with Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) with low glucose, containing 10% fetal bovine serum (FBS) (Gibco), 50.0 mg L⁻¹ gentamicin,

* Correspondence: betania.monteiro@gmail.com

100,000 U L⁻¹ penicillin G potassium, and 1.5 mg L⁻¹ amphotericin B, using a 10.0 mL syringe and a 25G needle.

The collected material was centrifuged at 694 G (2000 rpm) and 22 °C for 10 min, the supernatant was discarded and the pellet containing the cells was resuspended in a 15.0 mL of DMEM, plated in 25.0 cm² cell culture flasks, and incubated in an oven at 37 °C with 5% CO₂. Cultures were monitored daily using an inverted phase contrast microscope and the culture medium was replaced every 3 days.

At 80% confluence, the bottles were washed with phosphate buffered saline (PBS) and detached with a 1.0 mL solution of trypsin (Trypsin/EDTA, Cultilab, Brazil). After incubation for 5 min in an oven, the trypsin was inactivated with a DMEM complete medium. The suspension containing the cells was centrifuged at 694 G (2000 rpm) and 22 °C for 10 min in 15.0 mL Falcon tubes. The supernatant was discarded and the pellet resuspended in 5.0 mL of complete medium. After counting and a cell viability assay with Trypan blue, new plating was performed to allow cell expansion.

2.2 Osteogenic differentiation

After the fourth passage, the DMEM medium was supplemented with 10% FBS, 10⁻⁸ mol mL⁻¹ dexamethasone, 5.0 mg mL⁻¹ ascorbic acid 2-phosphate, 10.0 mmol L⁻¹ β-glycerophosphate, and incubated at 37 °C for 4 weeks. At days 7, 14, 21, and 30, the wells of the culture flasks were washed in PBS and stained with Alizarin red at room temperature for 5 min, washed with distilled water, and observed under an inverted phase contrast microscope for calcium deposition.

2.3 Immunophenotyping of MSCs

The fourth passage cells were incubation in 96-well plates at a concentration of 1.0 × 10⁵ cells per well. The samples were individually incubated with the primary antibodies (anti-CD45 clone 69 mouse, anti-CD90 clone Ox-7 mouse, anti-CD73 clone 5 F/B9 mouse, and anti-CD54 clone 1A29 mouse) (BD Bioscience, San Jose, CA, USA) for 30 min at 4 °C. Subsequently, the cells were washed with PBS and incubated with secondary antibody conjugated to Alexa 488 fluorochrome under the same conditions, according to the methodology described by Monteiro et al. (6). The samples were analyzed using a FACScan flow cytometer and CellQuest software.

2.4 MSC nanolabeling

The culture of the fourth passage was nanolabeled with Qtracker 655 (Invitrogen, São Paulo, Brazil) at a concentration of 2.0 μL for 2.0 × 10⁴ cells. The cells were incubated at 37 °C for 60 min and washed with DMEM three times.

Aliquots of labeled cells were taken 2 h, 24 h, and 7 days after nanolabeling for in vivo evaluation by fluorescence microscopy. After nanolabeling, cells were counted and

separated in centrifuge tubes at the concentration of 1.0 × 10⁷ cells/50 μL of PBS to be used immediately in the medical and surgical treatments.

2.5 Experimental animals

Thirty-six male New Zealand White rabbits, about 6 months of age and 3.0 kg in body weight were used. Prior to surgery the rabbits were put on solid-food fasting for 8 h, and preanesthetized with 0.5 mg kg⁻¹ acepromazine and 5.0 mg kg⁻¹ IM meperidine under intramuscular (IM) administration. They had their right forelimb shaved and trichotomized (hair removal) from the scapula to the metacarpals. Anesthesia was induced intravenously (IV) with 6.0 mg kg⁻¹ propofol and the maintenance of anesthesia was performed with isoflurane in semiclosed anesthetic circuit.

Previous surgical procedures were carried out using antibiotic prophylaxis with cephalexin (5.0 mg kg⁻¹, IM) and analgesia with morphine (2.0 mg kg⁻¹, subcutaneous) in the preoperative period and every 8 h postsurgery for 3 days.

Craniomedial approach to the radius was performed and a radial bone defect was surgically created by removing a bone segment of 1.0 cm length located 3.0 cm of the radiocarpal ulnar joint, using a circular saw. Both ends of the osteoperiosteal gap were washed with saline solution to remove the marrow from the distal and proximal fragments.

Nine rabbits were randomly assigned to four groups and the following treatments (Figure 1) were carried out:

- The control group (CG) received percutaneously administered 50.0 μL of PBS in the radial bone defect region, 24 h after lesion creation;
- Group 1 (G1) had the bone gap filled with absorbable gelatin sponge (Hemospon - Cremer - Sao Paulo, Brazil) soaked with 50.0 μL of PBS;
- Group 2 (G2) had the bone gap filled with absorbable gelatin sponge soaked with the MSC pellet (1.0 × 10⁷ cells/50.0 μL of PBS);
- Group 3 (G3) received percutaneously the MSC pellet (1.0 × 10⁷ cells/50.0 μL of PBS) in the bone gap region, 24 h after lesion creation.

At the end of the procedure, the muscle fascia and skin were sutured in both surgical sites. The animals were monitored for 50 days.

The evaluation included the animal's behavior (normal or apathy), body temperature, wound healing, conformation and weight bearing on the affected limb on the ground, and ambulation. Peripheral blood was collected 1 day before surgery and complete blood count was performed on days 1, 3, and 7.

Evaluation of radiographs from the medial/lateral and cranialcaudal (KV and 45 mA/s 0.08) views of the operated limbs was performed immediately postoperative and at

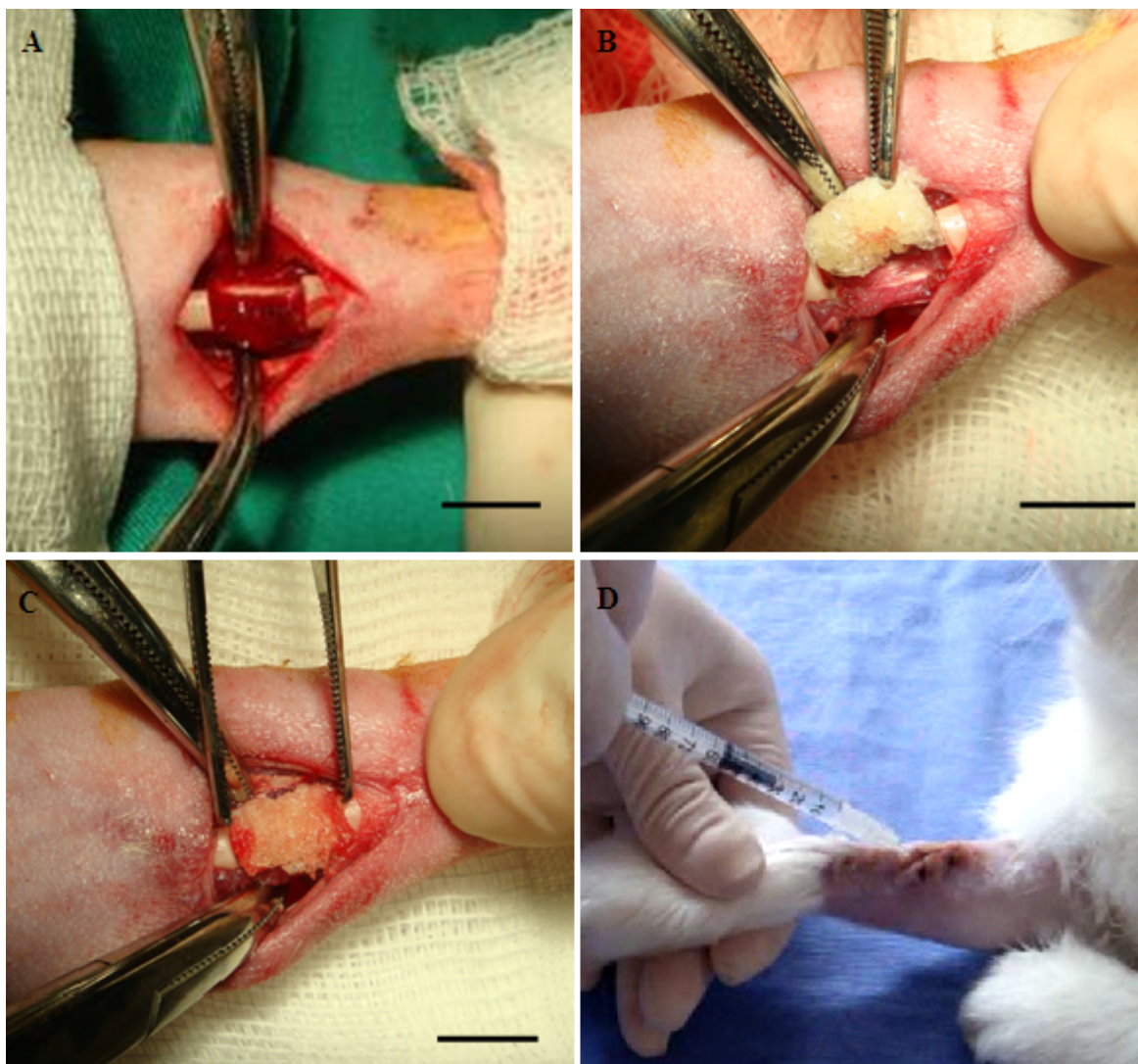


Figure 1. A radial bone defect was surgically created resulting in a bone gap of 1.0 cm. The animals were then subjected to different treatments: A) Control group, which had the gap filled with 50.0 μ L of PBS, percutaneously in the bone defect region 24 h after making the bone defect; B) Group 1, in which the gap was filled with absorbable sponge soaked with 50.0 μ L of PBS; C) Group 2, in which the gap was filled with absorbable sponge containing a cell pellet of 10^7 cells/50.0 μ L in PBS. D) Group 3, in which the defect was filled with a cell pellet containing 10^7 cells/50.0 μ L in PBS, percutaneously in the bone defect region 24 h after making the bone defect.

7, 21, 30, and 50 days, to assess the degree of bone gap filling. The bone gap filling was quantified as a percentage, depending on the radiopacity observed in the gap and compared with the image of day 0 of surgery, using the Image-Pro Plus software. Measurements were taken three times to give the mean values of the areas. To calculate the area, the total filling was considered as 100% and the value measured proportionally was calculated for percentage data by a mathematical formula.

2.6 Statistical analysis

Within each group, the body temperatures and leukocyte counts were analyzed using descriptive statistics and averages were compared.

The mean percentage of radial bone repair in each study period was calculated and subjected to ANOVA F test followed by Tukey's test in SAEG v 9.1 software (Sistema para Análises Estatísticas SAEG v. 9.1 - Universidade Federal de Viçosa, Viçosa, MG, Brazil) and Excel spreadsheet. The null hypothesis rejection level was established as 5% ($P \leq 0.05$).

3. Results

The amount of mononuclear cells obtained was always higher than the minimum recommended for initiating culture and cell viability (greater than 92.70%). On the first day, numerous cells of rounded shape were present

in the culture medium, not allowing the identification of the cell type (Figure 2a). Adherent cells with fibroblastoid morphology appeared 24 h after plating in complete DMEM medium (Figure 2b). After 3 days, the cultures reached 80% confluence (Figures 2c and 2d); then the first passage was performed. Intervals of 3–5 days were required for the other platings to attain the fourth passage.

Scanning electron microscopy confirmed in detail the fibroblastoid morphology of cells of all plates and slides from the early stages of culture (Figures 3a and 3b) and colony forming units in flasks with low cell density (Figure 3c). In the subsequent evaluations, confluence and arrangement of cultured cells were also observed (Figure 3d). Cell viability was always greater than 95.50% in the assessment of each passage.

Samples of each culture in the fourth passage were negative for CD45 expression (83.09%) and positive for CD54 (93.83%), CD73 (70.44%), and CD90 (98.82%), characterizing the cultured cells as MSCs (Figure 4).

Cultures subjected to osteogenic differentiation showed points of matrix calcification from the 7th day. Mineralized nodules containing cells with round morphology, similar to osteoblasts, were observed from the 14th day. High cytoplasmic red fluorescence was observed in nanolabeled

cells at all times of evaluation, both in the fresh samples and in the histologically processed pellet. With the remaining cells in osteogenic differentiation medium, most stem cells were differentiating into osteogenic cells and tended to 100% differentiation.

In the first 48 h postoperative, the rabbits avoided weight bearing on the affected limb, but fed normally. After 72 h, rabbits of G3 occasionally could bear weight on the affected limb and ambulation was normalized 96 h after surgery. The rabbits of the other groups started bearing weight on the affected limb after 96 h and only after that period they started ambulation.

There was no occurrence of hyperthermia after the treatment. The average patient's temperature, regardless of the experimental group, was 39.65 °C throughout the experimental period. No animal across the groups showed a fracture of the ulnar bone, wound dehiscence, or any sign of infection or clinical signs of rejection of the allogeneic cells and/or collagen sponge.

Leukocyte counts performed the day before surgery had a mean of $4.93 \times 10^3 \mu\text{L}^{-1}$, with no differences among the groups. On day 1, the means were $4.97 \times 10^3 \mu\text{L}^{-1}$ for CG, $5.15 \times 10^3 \mu\text{L}^{-1}$ for G1, $5.27 \times 10^3 \mu\text{L}^{-1}$ for G2, and $5.09 \times 10^3 \mu\text{L}^{-1}$ for G3, with no significant differences among the

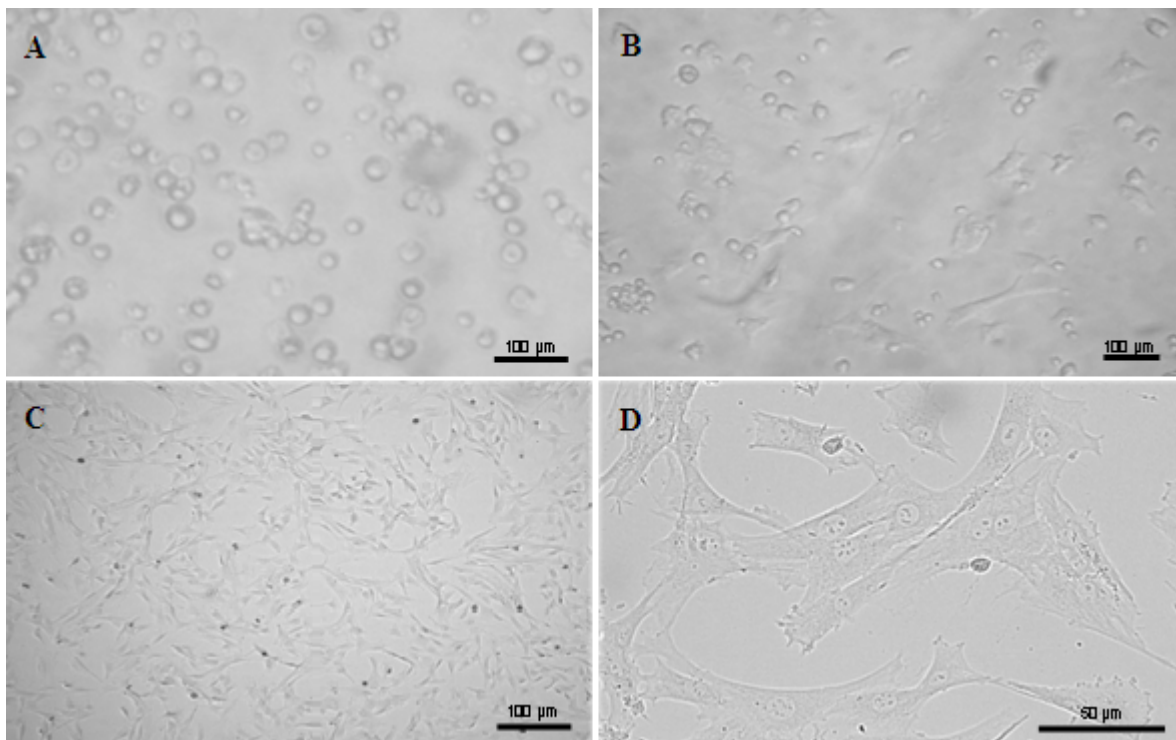


Figure 2. Photomicrographs captured by an inverted optical microscope: A) cells observed after collecting the bone marrow of mice exhibiting rounded morphology; B) 24 h after initiation of culture, with the appearance of the first fibroblastoid cells; C) the cell culture having a confluency greater than 80% and cells with fibroblastoid morphology and adhering to the substrate of the culture flask, 72 h after initiation of culture; D) featured presenting cells with fibroblastoid morphology of the cultured cell 72 h after initiation of culture.

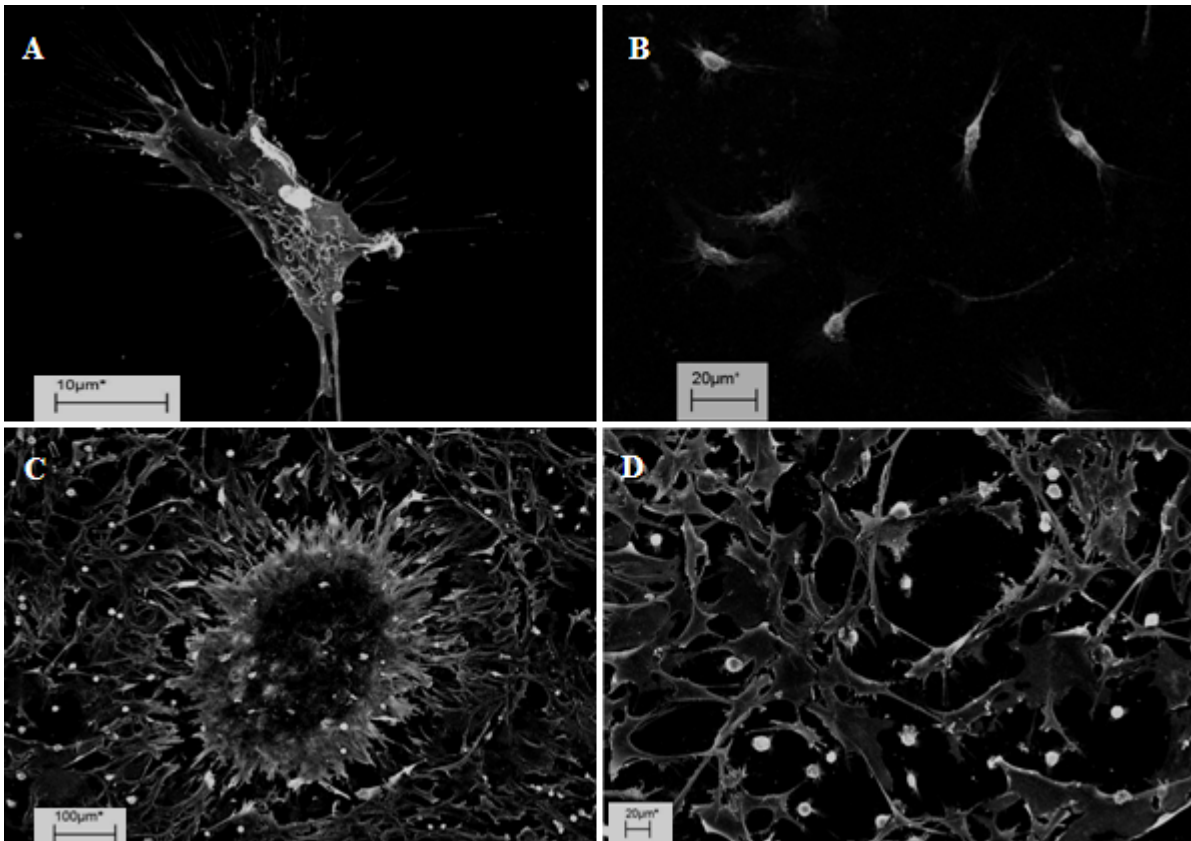


Figure 3. Photomicrographs in the scanning electron microscope: A) highlighting of fibroblastoid cells 24 h after initiation of culture; B) small cell density, with fibroblast cells 24 h after initiation of culture; C) cell growth from a colony forming units observed 48 h after the beginning of cell culture; D) cell culture having the highest confluence, cells with fibroblastoid morphology and adhering to the substrate of the culture flask, 72 h after initiation of culture. The rounded cells are cells that are in the process of mitosis, scrapping the adherence.

groups. In the remaining evaluation periods, the means for leukocyte counts were not significantly different from day 1, regardless of the experimental group, and the largest mean was $5.96 \times 10^3 \mu\text{L}^{-1}$, still within the normal range for the species.

Radiographs of the operated limbs in the immediate postoperative showed a radiolucent region with an average extension of 1.0 cm^2 . In G1 and G2, the collagen sponge had no positive radiodensity, which did not interfere in other subsequent evaluations, generating a confounding with the bone growth image. Radiographic evaluations after day 0 were compared with the first radiographs performed for each animal, and the average bone fill values were loaded on tables for statistical analysis.

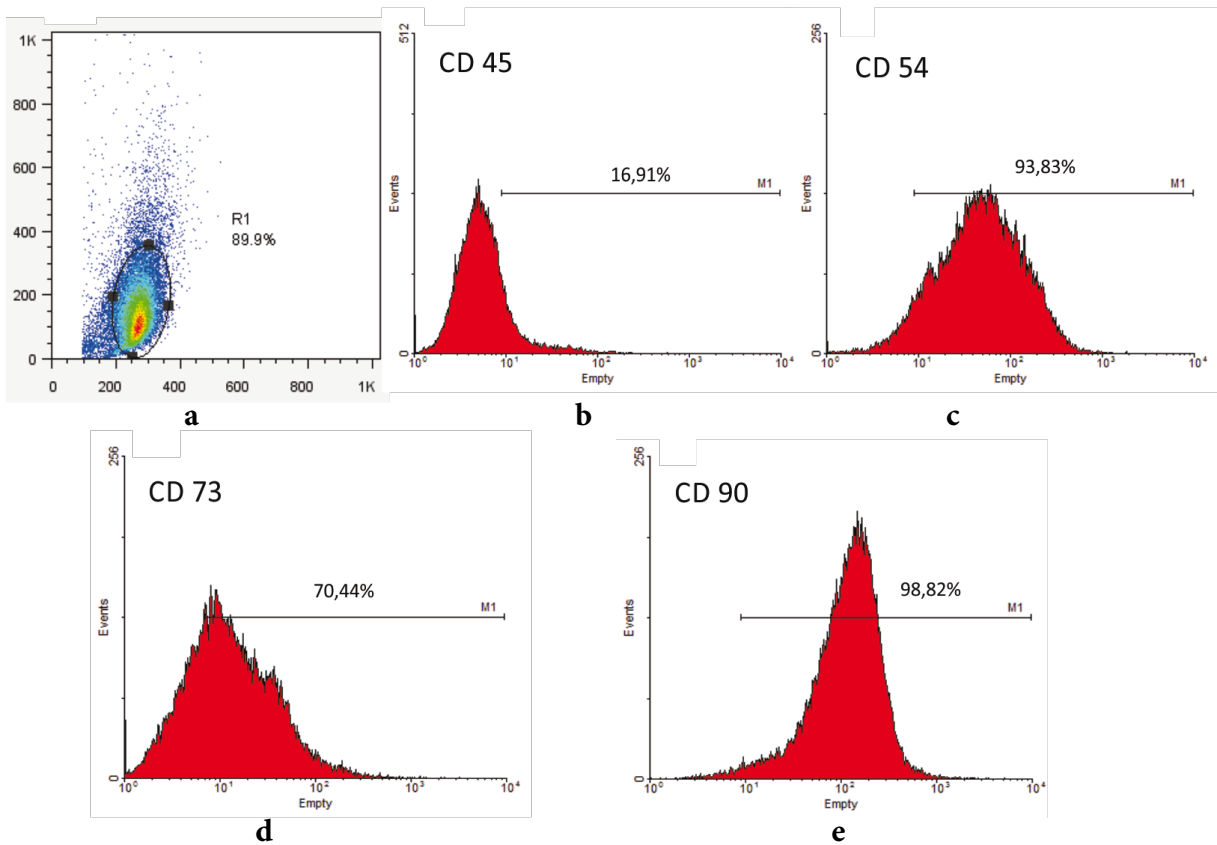
In all experimental groups, the radiographs showed increased radiopacity in the site of the created gaps. Mainly in CG and G3, bone growth occurred initially bridging the fracture gap from one end to the other, and this radiopacity was increased proportionally over the observation period. Rabbits of G1 and G2 sometimes showed radiopacity

started in the middle of the gap, indicating mineral matrix deposition above the osteoconductive membrane.

At day 7 of radiographic evaluation, the rabbits of GC had a bone filling lower than 5% of the total area (Figure 5a), the rabbits of G1 had an average bone filling of 7% (Figure 5b), the rabbits of G2 had an average bone filling of 9% (Figure 5c), and the rabbits of G3 had 18% bone filling (Figure 5d). At this time of the evaluation, there was a significant difference of G3 compared with the other groups, but no difference was found in bone filling of rabbits of G2 compared with CG and G1.

After 21 days, rabbits of G3 showed radiopacity of the radial bone defect region corresponding to a mean bone filling of 90%, without remodeling of the medullary canal. G2 rabbits showed 79.8% of bone filling, while rabbits of G1 showed bone filling of 71%. Rabbits of CG showed 46.3% of bone filling. At this time of the evaluation, there was also a significant difference of G3 in relation to the other groups. There was no significant difference between G2 and G1, but both groups differed from the control group.

4A



4B

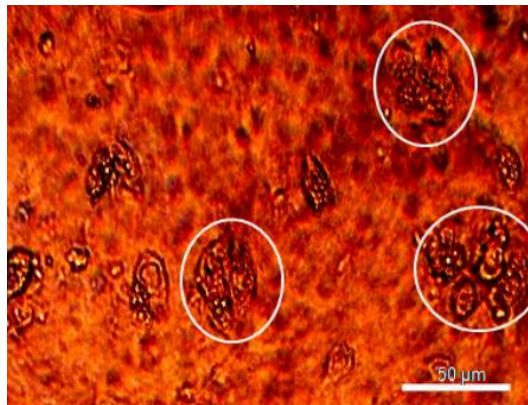


Figure 4. A) Evaluation of the frequency of CD45, CD54, CD73, and CD90 by flow cytometry, on bone marrow derived mesenchymal stem cells of rats. The fluorescence intensity of each surface marker in undifferentiated MSCs (white graphics) is compared with isotype controls (red graphics). The X-axis represents the fluorescence scale, the cells being positive when exceeding 10^1 . The Y-axis indicates the number of cells evaluated during the event. a) A dot plot showing the cell population selected for the study (R1), which represented 89.9% homogeneity. The culture samples showed a negative expression of CD45 at 83.09% (b), and 93.83% positive for CD54 expression (c), 70.44% of CD73 (d), and 98.82% of CD90 (e). B) The fourth cell culture passage submitted to osteogenic differentiation showing the presence of an extracellular matrix rich in calcium and rounded nodes, similar to osteoblasts in Alizarin Red staining at day 14.

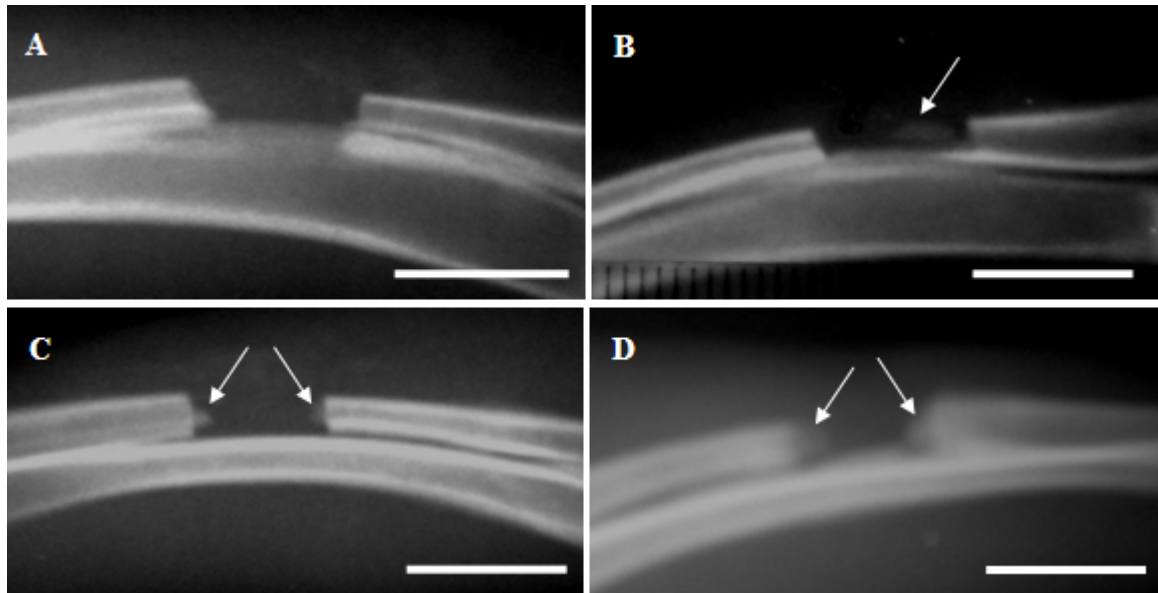


Figure 5. Radiographic images obtained at 7 days postsurgery demonstrating bone repair in each treatment: A) animals of the control group showing less than 5% bone filling of the total area; B) animals of group 1 showing an average bone filling of 7%; C) animals of group 2 with an average bone repair of 9%; D) animals of group 3 showing an average bone repair of 18% (scale equal to 1.0 cm).

At 30 days, seven rabbits of G2 had 90% of the bone gap filling and the other two rabbits showed an average filling of 79%, without the remodeling of the medullary canal. The average bone filling for G2 was 84.5%. All G3 rabbits, at this same evaluation time, showed a complete filling of the gap and an early formation of the medullary canal. G1 rabbits exhibited 80.7% bone filling, while rabbits of GC exhibited 50% bone filling, which arose from the edges of the gap. At this evaluation time, again there was a significant difference among the rabbits of G3 and those from the other groups of treatments; G2 and G3 did not differ, but were significantly different from the control group.

After 50 days of evaluation, all rabbits in groups G2 and G3 exhibited complete bone filling of the gap with an organization of the medullary canal (Figure 6c and 6d). G1 rabbits showed 97.43% of bone filling (Figure 6b) and GC showed 71.24% (Figure 6a). There was no significant difference among the three treatment groups, but they were significantly different from the control group.

4. Discussion

Adherent cells observed from 24 h of culture cannot be rated as MSCs, because some hematopoietic cells and fibroblasts can also be attached. Only by medium replacements and passages, these cells are removed and the heterogeneity of the culture gradually decreases, favoring only the growth of the stem cells of interest (7). After about 15 days, on the fourth passage, cellular homogeneity occurs and, for this reason, the characterization steps are performed from that time.

The expression of surface molecules CD54, CD73, and CD90 associated with a low expression of CD45 (16.91%) observed in this study, characterize the analyzed cells phenotypically as MSCs. The high levels of CD90 and CD54, both above 90%, are the most reliable findings to allow this conclusion, since, according to Ocarino et al. (8) and Kode et al. (9), hematopoietic cells and fibroblasts, which may be present in the culture, do not express such markers.

After 5 days in an osteogenic medium, the elongated fibroblastic cells changed appearance to a more rounded shape, resembling the morphology of the bone lineage. Also, the presence of a calcium-rich extracellular matrix, observed from day 7 and more evident with time, proved an early differentiation of cultured MSCs into osteoblasts, confirming the findings of Ocarino et al. (8). The fluorescent nanocrystals used in this study provided excellent cell labeling. They could be observed in the fresh samples obtained directly from the cell suspension after trypsinization, similar to that observed by Fischer et al. (10).

The immunoprivileged properties of mesenchymal stem cells have also been confirmed in this study, since the rabbits in the groups treated with these cells showed no clinical and hematological signs of animals with transplant rejection. These immunosuppressive properties have already been described *in vitro* by Nauta and Fibbe (3), Kode et al. (9), and Zhao et al. (11), and tested *in vivo* by Hernigou et al. (12) as autologous transplants, Monteiro et al. (6) as isogenic transplants, and Chuang

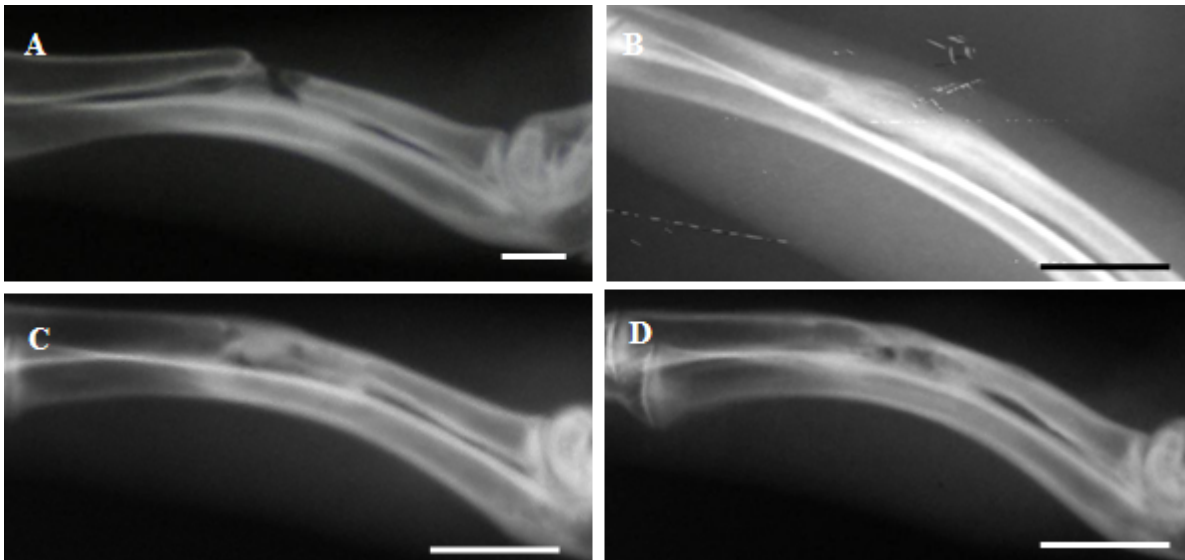


Figure 6. Radiographic images obtained at 50 days postsurgery demonstrating bone repair in each treatment: A) animals of the group control presenting a bone filling equivalent of 71.24% of the total area; B) animals of group 1 showing a filling average of 97.43%; C) animals of group 2 showing a complete bone repair of defects and early organization of the medullary canal; D) animals of group 3 showing a complete bone repair of defect and organization of the medullary canal (scale equal to 1.0 cm).

et al. (13) in allogeneic transplantation. Regarding xenogeneic transplantation, with results similar to those found by our research group, Chuang et al. (14) have succeeded in performing xenotransplantation of human stem cells in rats' calvaria.

In this study, it was also demonstrated that xenotransplantation of rat bone marrow-derived mesenchymal stem cells efficiently promoted bone repair of gaps created in the radius of rabbits, which were not immunosuppressed over the study period. Radiographic findings showed that the contribution of the cells in the bone repair process are similar to those reported by Shoji et al. (15) when treating femoral fractures in rats with human adipose-tissue derived mesenchymal stem cells. In both studies, the favorable effects of stem cells led to a functional recovery of the operated limb in less time when compared with the other experimental groups.

The sponge used inside the gap helped with bone healing by acting as an osteoconductive agent, in comparison with the control group. As described by Kwan et al. (16), gelatin or collagen sponges are considered an ideal three-dimensional matrix support to provide growth and cell expansion *in vivo*, as well as hyaluronic acid and chitosan, justifying what was observed for radiography. However, no differences were found between the groups in which the defect was filled with only the sponge and the group in which the defect was filled with the sponge soaked with cell pellet. When culturing cells in growth plates containing

the sponge, we observed that the cells did not adhere to the sponge pores. From this finding, it can be inferred that the sponge pore sizes are larger than that recommended in the literature to provide optimal cell growth. For this reason, the treatment with stem cells did not result in advantages in the process of bone repair. The collagen sponge used in the treatments has osteoconductive properties, but, because of its pore size, it was not a suitable platform for growth and expansion of stem cells used in the treatment of G2.

In this study, using clinical and radiographic evaluations, we concluded that xenotransplantation of rat bone marrow-derived mesenchymal stem cells percutaneously applied 24 h after the creation of a bone defect contributed positively to the repair of gaps in the radius of rabbits and did not cause rejection by the host.

Acknowledgments

The authors thank CNPq (National Council for Scientific and Technological Development), FUNADESP (National Foundation for Development of Private Higher Education), and FAPES (Research Support Foundation of the Espírito Santo) for their financial support and scholarships. They also thank the staff of the Laboratory of Cellular and Molecular Immunogenetics of the Federal University of Minas Gerais, the Laboratory of Biology and Control of Vectors and Hematozoa of the Federal University of Viçosa, and the Stem Cells and Cell Therapy Laboratory of the University of Vila Velha for the technical support.

References

1. Pittenger ME, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143–147.
2. Caplan, AI. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009; 217: 318–324.
3. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stroma cells. *Blood* 2007; 110: 3499–3506.
4. Wan CD, Cheng R, Wang H, Liu T. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. *Hepatobiliary Pancreat Dis Int* 2008; 7: 29–33.
5. Patel SA, Sherman L, Munoz J, Rameshwar P. Immunological properties of mesenchymal stem cells and clinical implications. *Arch Immunol Ther Exp* 2008; 56: 1–8.
6. Monteiro BS, Argôlo-Neto NM, Nardi NB, Chagastelles PC, Carvalho PH, Bonfá LP, Filgueiras RR, Reis AS, Del Carlo RJ. Treatment of critical defects produced in calvaria of mice with mesenchymal stem cells. *An Acad Bras Cienc* 2012; 84: 841–851.
7. Bydlowski SP, Debes AA, Maselli LM, Janz FL. Características biológicas das células-tronco mesenquimais. *Rev Bras Hematol Hemoter* 2009; 31: 25–35 (in Portuguese).
8. Ocarino NM, Boeloni JN, Goes AM, Silva JF, Marubayashi U, Serakides R. Osteogenic differentiation of mesenchymal stem cells from osteopenic rats subjected to physical activity with and without nitric oxide synthase inhibition. *Nitric Oxide* 2008; 19: 320–325.
9. Kode JA, Mukherjee S, Joglekar MV, Hardikar AA. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* 2009; 11: 377–339.
10. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS Jr. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009; 18: 683–691.
11. Zhao S, Wehner R, Bornhäuser M, Wassmuth R, Bachmann M, Schmitz M. Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. *Stem Cells Dev* 2010; 19: 607–614.
12. Hernigou PH, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone marrow grafting for nonunions – influence of number and concentration of progenitor cells. *J Bone Joint Surg Am* 2005; 87: 430–437.
13. Chuang CK, Wong TH, Hwang SM, Chang YH, Chen GY, Chiu YC, Huang SF, Hu YC. Baculovirus transduction of mesenchymal stem cells: in vitro responses and in vivo immune responses after cell transplantation. *Mol Ther* 2009; 17: 889–896.
14. Chuang CK, Lin KJ, Lin CY, Chang YH, Yen TC, Hwang SM, Sung LY, Chen HC, Hu YC. Xenotransplantation of human mesenchymal stem cells into immunocompetent rats for calvarial bone repair. *Tissue Eng Part A* 2010; 16: 479–488.
15. Shoji T, Li M, Mifune Y, Matsumoto T, Kawamoto A, Kwon SM, Kuroda T, Kuroda R, Kurosaka M, Asahara T. Local transplantation of human multipotent adipose-derived stem cells accelerates fracture healing via enhanced osteogenesis and angiogenesis. *Lab Invest* 2010; 90: 637–649.
16. Kwan MD, Slater BJ, Wan DC, Longaker MT. Cell-based therapies for skeletal regenerative medicine. *Hum Mol Genet* 2008; 17: R93–R98.