Reconstruction of Large Cranial Defects in Nonimmunosuppressed Experimental Design With Human Dental Pulp Stem Cells

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The main aim of this study is to evaluate the capacity of human dental pulp stem cells (hDPSC), isolated from deciduous teeth, to reconstruct large-sized cranial bone defects in nonimmunosuppressed (NIS) rats. To our knowledge, these cells were not used before in similar experiments. We performed two symmetric full-thickness cranial defects (5 × 8 mm) on each parietal region of eight NIS rats. In six of them, the left side was supplied with collagen membrane only and the right side (RS) with collagen membrane and hDPSC. In two rats, the RS had collagen membrane only and nothing was added at the left side (controls). Cells were used after in vitro characterization as mesenchymal cells. Animals were euthanized at 7, 20, 30, 60, and 120 days postoperatively and cranial tissue samples were taken from the defects for histologic analysis. Analysis of the presence of human cells in the new bone was confirmed by molecular analysis. The hDPSC lineage was positive for the four mesenchymal cell markers tested and showed osteogenic, adipogenic, and myogenic in vitro differentiation. We observed bone formation 1 month after surgery in both sides, but a more mature bone was present in the RS. Human DNA was polymerase chain reaction-amplified only at the RS, indicating that this new bone had human cells. The use of hDPSC in NIS rats did not cause any graft rejection. Our findings suggest that hDPSC is an additional cell resource for correcting large cranial defects in rats and constitutes a promising model for reconstruction of human large cranial defects in craniofacial surgery.

Key Words: Human dental pulp stem cells, adipogenic differentiation, cranial defect, nonimmunosuppressed rats

Cranial defects are a relatively common complication that can arise secondary to trauma, surgery, infection, neoplastic, or congenital malformation such as aplasia cutis (AC), and its reconstruction is a challenge in craniofacial surgery. Large cranial defects, which are not rarely observed in AC,[1–3] are associated with high morbidity and mortality in the neonatal period and therefore, their treatment is important not only to improve the aesthetic appearance, but mostly important for the reestablishment of the rigid protection of the underlying brain.[4,5]

Transplant of bone autograft remains the currently used method for reconstruction of cranial bone loss; however, it is inadequate for large defects and implicates several risks, including donor site morbidity, graft or flap failure, rejection, or infection.[6,7] Development of more effective methods for calvaria reconstruction, particularly for large defects, will certainly have major implications for the repair of cranial defects in AC or others with known genetic or other environmental causes.

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Use of mesenchymal stem cells (MSC) for bone reconstruction has been an active growing area that can facilitate bone healing in difficult circumstances, and possibly in the near future, it will replace conventional therapeutic modalities for the repair of large cranial defects. These cells have self-renewing properties and are able to differentiate into one or many different specialized cell types under controlled in vitro conditions. They can be obtained from many tissues such as bone marrow,[8] dental pulp,[9] adipose tissue,[10] and others.[11] Several studies have shown the efficiency of bone marrow MSC application in the closure of critical size defects in murine calvarium.[12,13] However, MSC from other tissues have not yet been tested in similar studies. The use of human dental pulp stem cells[7] (hDPSC) is of great interest in bone reconstruction because they can be easily isolated and expanded in culture; moreover, they have shown in vitro and in vivo multipotential plasticity.[8,9,15,16] These cells seem to have immunosuppressive activity that could have potential clinical applications in allogeneic in vivo stem cell transplantation, particularly for calcified tissue reconstruction.[17]

Until now, transplantation of undifferentiated hDPSC with collagen membrane has never been used to correct cranial defects and human stem cells have never been transplanted in non immunosuppressed animals. Therefore, to address these questions, we evaluated if undifferentiated hDPSCs growing on a collagen membrane (CM) are able to reconstruct large-sized cranial bone defects created in nonimmunosuppressed (NIS) rats. Before transplantation, hDPSC were characterized using MSC markers and their in vitro differentiation potential was addressed by the production of osteoblasts and other cell types.

Materials and Methods

Subjects and Human Dental Pulp Culture

A 6-year-old healthy subject was enrolled in this study after obtaining informed signed consent by their parents in accordance to the guidelines of the Ethical Committee of Institute of Biosciences, University of São Paulo. The dental pulp was extracted from normal nonexfoliated deciduous tooth under local anesthesia. The cells were obtained from the dental pulp as previously described.[16] The growing culture of hDPSC was maintained in Dulbecco's modified Eagle's medium/Ham's F12 (1:1; Invitrogen, Carlsbad, CA) supplemented with 15% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 2 mM nonessential amino acids. The cells were maintained semiconfluent to prevent the differentiation and were passed every 4 to 5 days with the medium refreshed daily. The hDPSC were incubated at 37°C in a 5% CO_2 and a high-humidity environment.

Characterization of Human Dental Pulp Stem Cells

The following antibodies against cell surface molecules and their respective isotype controls were used for flow cytometry analysis: monoclonal anti-human, CD29, CD45 conjugated with fluorescein isothiocyanate conjugated, CD34 (Becton Dickinson, Franklin Lakes, NJ), and SH2 (CD105) (Serotec, Oxford, UK). Antibodies against SH3 and SH4 were from Case Western Reverse University Cleveland, OH. Approximately 10^6 cells were incubated with primary antibody for 30 minutes at 4°C, washed in phosphate-buffered saline + 2% fetal bovine serum and 1 μM sodium azide (buffer) followed by addition of secondary anti-mouse-polyethylene-conjugated antibody according to the manufacturer's instructions (Guava Technologies, Hayward, CA). Control samples were incubated with phosphate-buffered saline and with secondary antibody. Flow cytometry analysis was performed on Guava EasyCyte with the Guava ExpressPlus software (Guava Technologies).

The cells were submitted to osteogenic, adipogenic, and myogenic in vitro differentiation according to routine protocols.[18–21] The von Kossa staining method was used at the 21st day after induction of osteogenic differentiation of hDPSC. The cells induced to adipogenic differentiation were stained at day 12 with oil red-O.[20] To characterize myogenic differentiation, the following antibodies were used: monoclonal anti-α-sarcomeric anticlone 5C5 (developed in mouse; Sigma, St. Louis, MO); monoclonal antimyosin skeletal (developed in rabbit; Sigma); and monoclonal antititin clone T11 (developed in mouse; Sigma). The secondary antibody was goat anti-mouse, CY3 (CyDye) and fluorescein isothiocyanate conjugated (labeled goat anti-rabbit; Santa Cruz Biotechnology, Santa Cruz, CA). Respective negative controls were also performed (data not shown).

Animals

Four-month-old male NIS Wistar rats, with body weight 320 to 420 g, were used for this study. The Animal Research Ethics Committee at the University of São Paulo approved the experimental

protocol. The animals were kept in ventilated stands (Alesco, São Paulo, Brazil), in standardized air and light conditions at a constant temperature of 22°C with a 12-hour light/day cycle. They had free access to tap drinking water and standard laboratory food pellets.

Surgery and Transplantation Procedure

To evaluate the potential of the hDPSC to reconstruct large cranial defects, we have done two symmetric full-thickness cranial defects of 5 × 8 mm[22] size on each parietal region of eight animals (Fig 1) and the left side (LS) was used as a control of the right side (RS). The cranial defect was done with a drill with micromotor under constant irrigation with sterile physiological solution to prevent overheating of the bone. The underlying dura mater was left undisturbed. Two rats were used as controls: they received CM only at the RS, and they had only the cranial defect at the LS. Six NIS animals (experimental group) received the CM only on the LS, whereas CM with undifferentiated hDPSC was placed on the RS.

The animals were anesthetized with an intraperitoneal injection (0.3 mL/100 g of body weight) with a combination of ketamine hydrochloride (5%) and xylazine (2%). The head of the rats was positioned in a cephalostat during the operative procedure. A midline skin incision was performed from the nasofrontal area to the external occipital protuberance. The skin and underlying tissues, including the periosteum and the temporalis muscles were reflected laterally to exposure the full extent of the calvaria.

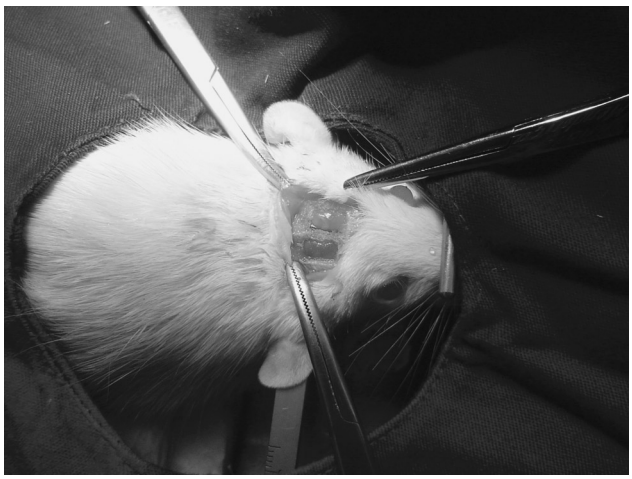

Fig 1 Cranial defects (5 × 8 mm) induced in the parietal cranial bones in the rat.

For the transplantation procedure, 10^6 undifferentiated hDPSC were seeded onto collagen membrane (Oral Medica, São Paulo, Brazil) (CM, 8 mm × 5 mm) in six-well plate each of 35 mm (Corning, Corning, NY). The wells were supplemented with 2.5 mL of basal culture medium and the cells were incubated approximately 24 hours at 37°C in 5% CO_2 before transplantation to adhere onto the CM.

The flap of each animal was closed with nylon 4 sutures (Ethicon, São Paulo, Brazil). The experimental group was euthanized 7 days (n = 1), 20 days (n = 2), 30 days (n = 2), and 60 days (n = 1) after surgery with inhaled CO_2. The animals of the control group (n = 2) were euthanized after 120 days.

Histologic Preparation

Samples of the LS and RS of each animal were prepared for histologic analysis. The studied tissue samples were fixed in 10% formalin for 24 hours, decalcified in 5% formic acid for 48 hours, and paraffin-embedded. The 5-μm sections were stained with hematoxylin and eosin and examined under a light microscope, Axiovert 200 (Carl Zeiss, Jena, Germany).

Analysis of the Presence of Human Cells in the New Bone

Tissues were scrapped from histologic section slides for DNA extraction. Material was obtained from the LS (only membrane) and RS (CM + hDPSCs) critical calvarium defects of rats at 2 months after transplantation. DNA was extracted according to the tissue protocol of the QIAamp DNA Mini KIT (Qiagen, Hilden, Germany). For amplification of the human DNA, polymerase chain reaction reactions were performed with exon 8 of COL18A1 as previously reported.[23] Specific rat primer was used for amplification of rat DNA (*Gapdh* gene). Two rounds of polymerase chain reaction of 35 and 30 cycles were respectively performed.

RESULTS

Characterization and In Vitro Differentiation of Human Dental Pulp Stem Cells

We isolated the population of hDPSC, which were mainly (96% ± 3%) positive for the four MSC markers tested (SH2, SH3, SH4, and CD29 antigens) and negative for hematopoietic cell lineage markers (CD34 and CD45). Microscopically, these cells showed typical fibroblast-like MSC morphology

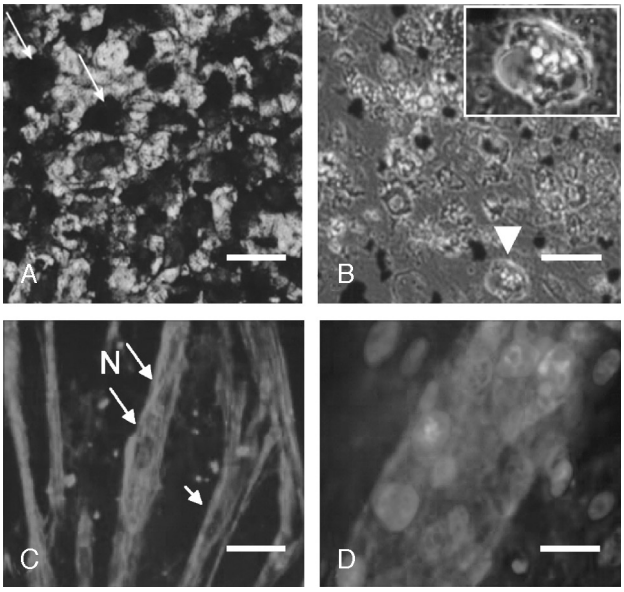

Fig 2 In vitro differentiation of human dental pulp stem cells (hDPSC). (A) von Kossa staining revealed calcified extracellular matrix (arrows) in hDPSC cultured in osteogenic medium for 21 days. (B) Oil red O staining of hDPSC cultured in adipogenic medium (arrowhead). Inset in higher magnification showed accumulation of lipid-filled droplets within the differentiated hDPSC. The cells cultured in control osteogenic and adipogenic mediums remained von Kossa and Oil red O-negative, respectively (data not shown). Myogenic differentiation observed in hDPSC: immunofluorescence revealed (C) tropomyosin and (D) actin-positive cells at 60 days after induction of differentiation. Arrows (C) and DAPI (blue), DNA-binding dye (D) staining indicate nuclei (N) in multinucleated muscle fibers. Negative control was performed with respective isotype controls for undifferentiated hDPSC (data not shown). Bars = 50 μm.

(data not shown) and under appropriate conditions, they differentiate into osteoblasts, myoblasts, and adipocytes (Fig 2).

Bone Reconstruction After Human Dental Pulp Stem Cell Transplantation

We have evaluated the bone healing process at the cranial defects by histologic analysis at the seventh, 20th, 30th, and 60th days after surgery in the animals that were transplanted with cells, whereas evaluation of the cranial defect in a control animal was done at the 120th day. None of the animals died of infection or any other complication.

At 7 days postsurgery, the gap at the LS, where only the CM was used, was filled by loose connective tissue showing diffuse and intense chronic inflammatory infiltrate rich in foam macrophages and remnants of the CM were still present (Fig 3A). The same scenario was observed regarding the group in which the CM was placed together with hDPSCs (RS), but the presence of lymphocytes was reduced and no signs of CM were seen (Fig 3B). At day 20 postsurgery, both sides at the defect area showed a mixture of immature cortical bone and granulation tissue in the two animals analyzed. On the RS, the bone was slightly more mature with some lamellae formation and more inconspicuous granulation tissue (data not shown). One month postsurgery, the regeneration process was more advanced and both sides were totally filled by new bone, which was partially immature and lamellar. The newer formed bone was fused to the remnant bone. In the LS wound, it was still possible to notice granulation tissue, whereas in the RS, which contains the hDPSC, the bone formation appears to be denser and more mature (Fig 3C–D). The cranial defect was apparently healed at 60 days postsurgery in the animal analyzed (Fig 3E–F).

The cranial defect of 5 × 8 mm in control animals was patent at the 120th day postsurgery (data not shown).

DNA amplification using human-specific primers was successful only with DNA derived from the RS cranial defect. In the material derived from the LS cranial defect, positive DNA amplification was only obtained with the use of the rat-specific primers (Fig 4).

Discussion

We showed that hDPSC used in the present study express MSC markers and can successfully differentiate into osteoblasts and skeletal muscle cells. We also demonstrated adipogenic differentiation of these cells, which was not reported previously for hDPSC.[9,14,16] We were able to isolate and differentiate into several cell types the hDPSC from three other unrelated individuals who were respectively 5, 7, and 10 years of age (data not shown), thus reinforcing that the protocol that we used[16] is highly reproducible and that pluripotent cells of children up to 10 years old are successfully obtained.

Our results showed that hDPSC with CM induced new bone formation in the calvaria critically sized defects with formation of a lamellar bone with a very efficient union of the new bone with the adjacent calvarium in the NIS rats. Although our data should be considered as preliminary, we observed that the use of hDPSC with CM leads to a very homogenous bone

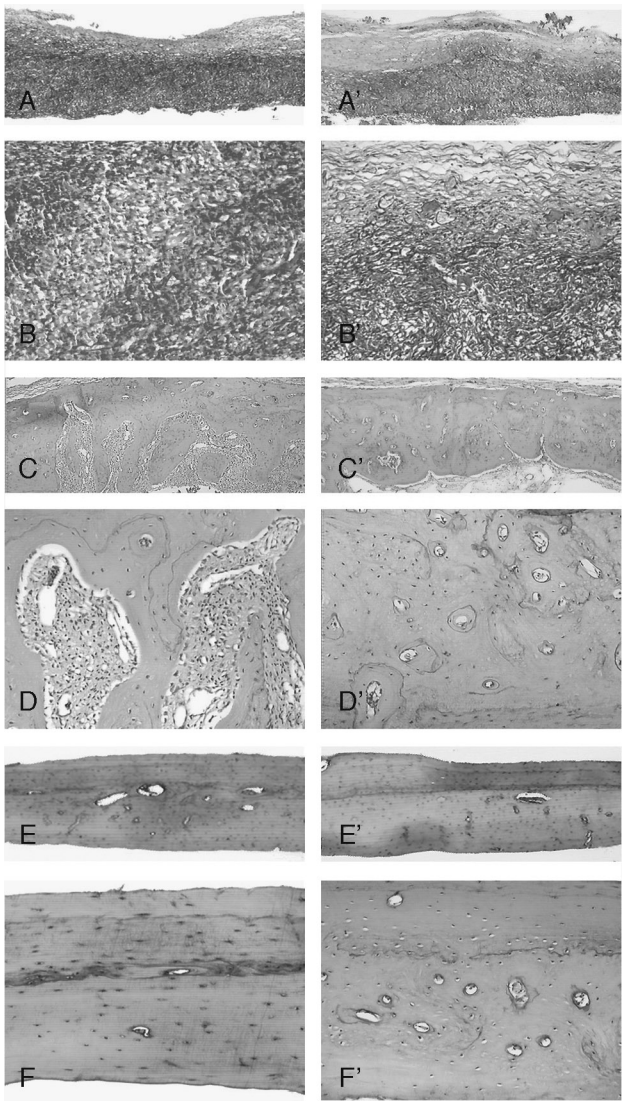

Fig 3 Comparative histologic analysis of the cranial defect reconstruction at 7 (A–B), 30 (C–D), and 60 (E–F) days postsurgery, respectively. (A′, C′, E′) Closed with collagen membrane (CM) (magnifications 10×, 25× and 25×, respectively, A′, C′, E′) showed higher magnification (100× B′, D′, F′). Closed with CM + hDPSC (magnifications 10×, 25× and 25×, respectively, B′, D′, F′) showed higher magnification (100×). Histologic analysis of the healing in the cranial defect at 7 (A–B), 30 (C–D), and 60 (E–F) days postsurgery. (A–F) The side where CM only was applied, whereas A′ to F′ represent the side where CM + hDPSCs were used. A, A′, C, C′, E, E′ 100×; B, B′, D, D′, F, F′ at 250× magnification. Note at C′ and D′ a more well-formed and mature bone as compared with C and D.

formation process in the experimental animals, suggesting that this is a promising approach. The defect was also healed with the use of CM only, but the ossification process was evidently delayed when compared with the use of CM and hDPSC (Fig 3A–F). These results also support that the use of CM alone does not promote an adequate bone regeneration that can be used as a bone graft substitute.[24]

Mankani et al[25] conducted a study with human bone marrow stem cells (hBMSCs) and hydroxyapatite/tricalcium phosphate (HA/TCP) in immunocompetent mice and a significant bone formation in 75% of the animals transplanted with hBMSCs and HA/TCP in 6 weeks was achieved. The cranial defects induced in this study and in ours are comparable, and therefore we could suggest that human mesenchymal cells either of bone marrow or dental pulp seem to be very efficient to reconstruct large cranial defects. It is important to note that hDPSCs have several advantages when compared with MSC derived from other sources; the method for their isolation is not invasive and they can be rapidly expanded in vitro.

Krebsbach et al[26] demonstrated that murine BMSCs were capable of closing critically sized murine calvarial defects when placed in conjunction with a collagen carrier 2 weeks after transplantation. However, these transplants failed to form a union with the adjacent mouse skull, which was attributed to an interference of the periosteum that was not totally removed. It has been suggested that the use of CM associated with hBMSCs is not appropriate for successful new bone formation, because these cells also require a mineral matrix, a BMP-expressing nonmineral matrix, or production of BMP-2 by genetically engineered cells.[25] However, our results contradict these previous data, because we obtained a very efficient healing bone process with the use of collagen membrane and hDPSCs. Therefore, our data support the hypothesis that collagen membranes are a good scaffold, and they can represent advantages in relation to HA/TCP because they facilitate cell adhesion and bone-forming cells into the defect site. Indeed, hydroxyapatite granules are slowly reabsorbed and could be surrounded by a connective tissue capsule that may affect cell migration and velocity of new bone formation into the defect site as well as the graft stability.[27] We cannot exclude the possibility that the success of the healing process that we obtained is related to the type of the collagen membrane used.[28] Collagen membranes are also technically easier to place into a bone defect than are the HA/TCP particles. It is still possible that the origin of the MSC used interferes in the bone regeneration processes. Although both hDPSC and hBMSC showed high plasticity in vitro or in vivo, it has yet not been compared if they present any

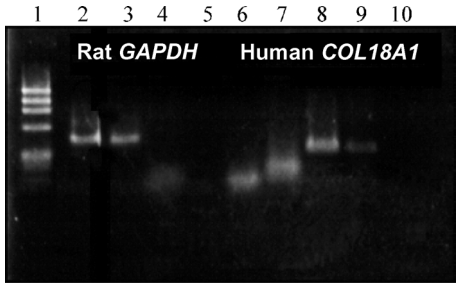

Fig 4 Gel eletrophoresis of polymerase chain reaction (PCR) products generated through PCR amplification of DNA extracted from both cranial defects from a rat at 60 days after surgery: 1) molecular weight ϕx174 RFDNA/Hae III fragments (Invitrogen); 2–4: PCR products after amplification with Gadh rat-specific primers, where 2 = rat DNA as control of the reaction, 3 = DNA from the cranial defect with collagen membrane (CM) only, 4 = DNA from the cranial defect with CM + human dental pulp stem cells (hDPSC); 5 = negative control for the PCR reaction; 6–9: PCR products after amplification with COL18A1 human-specific primers where 6 = rat DNA as control of the reaction, 7 = DNA from the cranial defect with CM only, 8 = DNA from the cranial defect with CM + hDPSC, 9 negative control for the PCR reaction.

in vivo difference in proliferation or in differentiation properties.

We did not use any protocol for immunosuppression in our experimental animals; however, no clinical symptoms of human cell rejection were observed in the recipient rats. The demonstration that the new bone formed in our experimental rats had human cells adds evidence that the animals did not reject the hDPSC. It will be important to address if these human cells do not trigger the immunologic system of the recipients. It has already been shown that MSC do not induce T-cell alloreactivity and display an immunoregulatory capacity by suppressing T-cell responses in vitro and in vivo.[17,29,30] These results thus have important future implications in terms of allogeneic stem cell therapy.

CONCLUSION

Herein we showed the use of hDPSC together with CM as a promising strategy for in vivo bone tissue reconstruction and their use might provide an option to repair human large cranial bone defects. It is possible that hDPSCs can be used as an alternative source to hBMSC in tissue bone regeneration. Furthermore, our results indicate that hDPSC apparently do not stimulate allogeneic graft rejection by the recipient organism. We also demonstrated that these cells can differentiate efficiently into adipocytes and skeletal muscle cells. These two types of cell differentiation together with bone formation are the essential elements for tissue reconstruction of different craniofacial defects. Hence, the hDPSC appear as a potential source of stem cells to be used in plastic surgery, particularly among craniofacial anomalies.

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