Stem Cells from Human Exfoliated Deciduous Teeth (SHED) Differentiate in vivo and Promote Facial Nerve Regeneration

Larissa Vilela Pereira1, Ricardo Ferreira Bento1,2, Dayane B. Cruz3, Cláudia Marchi2, Raquel Salomone1, Jeanne Oiticicca1,2, Márcio Paulino Costa4, Luciana A. Haddad3, Regina Célia Mingroni-Netto3, and Heloisa Juliana Zabeu Rossi Costa1

Abstract

Post-traumatic lesions with transection of the facial nerve present limited functional outcome even after repair by gold-standard microsurgical techniques. Stem cell engraftment combined with surgical repair has been reported as a beneficial alternative. However, the best association between the source of stem cell and the nature of conduit, as well as the long-term postoperative cell viability are still matters of debate. We aimed to assess the functional and morphological effects of stem cells from human exfoliated deciduous teeth (SHED) in polyglycolic acid tube (PGAt) combined with autografting of rat facial nerve on repair after neurotmesis. The mandibular branch of rat facial nerve submitted to neurotmesis was repaired by autograft and PGAt filled with purified basement membrane matrix with or without SHED. Outcome variables were compound muscle action potential (CMAP) and axon morphometric. Animals from the SHED group had mean CMAP amplitudes and mean axonal diameters significantly higher than the control group ($p < 0.001$). Mean axonal densities were significantly higher in the control group ($p = 0.004$). The engrafted nerve segment resected 6 weeks after surgery presented cells of human origin that were positive for the Schwann cell marker (S100), indicating viability of transplanted SHED and a Schwann cell-like phenotype. We conclude that regeneration of the mandibular branch of the rat facial nerve was improved by SHED within PGAt. The stem cells integrated and remained viable in the neural tissue for 6 weeks since transplantation, and positive labeling for S100 Schwann-cell marker suggests cells initiated in vivo differentiation.

Keywords
facial nerve, facial nerve regeneration, human exfoliated deciduous teeth stem cell (SHED), autograft, nerve repair, polyglycolic acid tube

Introduction

The facial nerve plays important roles in physiological and social functions. Therefore, facial paralysis may lead to physical and psychological incapacitation. Severe facial nerve lesions (neurotmesis) do not result in spontaneous recovery, and functional outcomes are still poor even after the best surgical repair.

Autografting, with or without neurotube association, remains the clinically accepted gold-standard technique in cases of extensive facial nerve damage for which a single anastomosis is not feasible. Thus, stem cell transplantation has been associated with standard methods yielding more encouraging results. Positive effects with bone marrow stem cells (BMSC) on facial nerve regeneration have been demonstrated, but there have been only a few studies that...
employed stem cells from dental pulp for facial nerve repair. Dental pulp appears to be an alternative and less invasive source of stem cells when compared with BMSC. Stem cells from human exfoliated deciduous teeth (SHED) and from the pulp of permanent teeth (DPSC) feature a population of multipotent, self-renewing mesenchymal stem cells that actively secrete a broad spectrum of trophic and immunomodulatory factors. The main advantages of using SHED are that they have higher proliferation rate than DPSC and they can be easily obtained from deciduous teeth that are routinely extracted in childhood and generally discarded without any ethical concerns.

In the current study, we employed nerve autografting combined with SHED and a polyglycolic acid tube (PGAT). Our specific aims were to compare the functional and histological outcomes of the facial nerve, and to evaluate the presence and phenotype of the exogenous cells in the autografted nerve 6 weeks after transplantation. An objective comparison was performed to assess the compound muscle action potential (CMAP) and the axonal morphometry variables. We observed the highest CMAP amplitudes and axonal diameters in the SHED group. Our study also reveals unprecedented results on the in vivo maintenance and integration of SHED, which differentiated in vivo into Schwann-like cells in the graft along the 6 weeks. The superior characteristics of the conduit and extracellular membrane components employed were likely related to the maintenance of viable and differentiated cells at the end of the study.

Materials and Methods

Animals

Wistar rats were obtained from the animal facility at the University of São Paulo Medical School. All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of University of São Paulo, São Paulo, Brazil, and approved by Administration Committee of Experimental Animals, University of São Paulo, São Paulo, Brazil (no. 075/14). Seventeen adult males weighing between 250 and 300 g were used in the experimental surgery. Anesthesia for surgical procedures consisted of the intraperitoneal injection of ketamine (4 mg/100 g) and xylazine (1 mg/100 g). The animals received a single dose of intramuscular penicillin G potassium (50,000 U/kg) in the immediate post-surgical period. Sacrifices were carried out with an anesthetics overdose.

Stem cells

SHED lines were isolated from normal exfoliated human deciduous teeth collected from children aged 6 to 8 years old with written informed consent obtained from legally representative(s) for anonymized patient information to be published in this article and under approved guidelines set by the Ethics Committee, Biosciences Institute, University of São Paulo, Sao Paulo, Brazil (no. 711.639/14).

The pulp was separated from the remnant crown and then digested in a solution of Tryple Express (Thermo Fisher Scientific, Waltham, MA, USA). After digestion, cells were maintained in 6-well culture plates containing DMEM/F12 supplemented with 15% FBS (x), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 2 mM non-essential amino acids (Thermo Fisher Scientific). After SHED lines were established, cells were washed with PBS (0.0 M), dissociated with Tryple Express for 7 min and cells were seeded in 25 cm² culture flasks (Corning). Cells were kept at 37°C in a 5% CO₂ incubator and maintained in semi-confluence to prevent differentiation. Medium was refreshed every 2 days, and passages were done every 4 days.

Before the transplantation experiments, cellular characterization was performed with the purpose of confirming their multipotent features. This was performed using two approaches: through immunophenotypic characterization by flow cytometry, and by means of cell differentiation. Immunophenotypic characterization of SHED was done by flow cytometry (FACS Aria II - BD Biosciences, San Jose, CA, USA). Cells were harvested with Tryple Express, and resuspended to 10⁵ cells in 100 μL of PBS and incubated with the conjugated antibodies (1:500) for 1 h. The recommended panel was used for the characterization of multipotent mesenchymal cells by means of flow cytometry. The panel is composed of specific antibodies to identify cell markers of mesenchymal origin (CD29-PerCP, CD73-PE, CD90- Alexa700, CD105-PE, and CD166-PE), and hematopoietic and endothelial origin (CD31- PE, CD34-PerCP-Cy5, and CD45-FITC). Only cultures that were positive regarding the expression of characteristic markers of cells of mesenchymal origin and negative for the expression of markers of hematopoietic and endothelial cells were used in the experiments.

Analysis of in vitro differentiation was performed in order to verify the differentiation capacity of SHED. The established lines were submitted to chondrogenic, adipogenic, and osteogenic differentiations. A density of 5×10⁵ cells/cm² were plated in 24-well plates, and, after 24 h of culture in regular medium, SHED populations were washed with 1× PBS and treated with StemPro® Osteogenesis Differentiation Kit (Thermo Fisher Scientific) for osteogenic differentiation, StemPro® Adipogenesis Differentiation Kit (Thermo Fisher Scientific) for adipogenic differentiation, and StemPro® Chondrogenesis Differentiation Kit (Thermo Fisher Scientific) for chondrogenic differentiation. All experiments were done in technical triplicates. Only SHED lines capable of chondrogenic, adipogenic, and osteogenic differentiation were used in subsequent experiments.

The cells were then cryopreserved and the tube was thawed by gentle shaking at 37°C and decontaminated by immersion in 70% ethanol. In laminar flow (sterile environment), the tube was opened and the contents (approximately 2 ml) containing the SHED were transferred to a 15 ml Falcon tube. Then, culture medium, along with SHED, was added to the Falcon tube in order to remove the
cryoprotective agent (DMSO). The Falcon tube was centrifuged for about 5 min at a rate of 100×g, at a temperature between 22 and 25°C. Again, in laminar flow, the supernatant was discarded and the cell pellet was resuspended in 4 ml of culture medium. This material was transferred to a T-25 culture bottle and incubated in a CO₂ incubator at 37°C. Within 24 h, the cells adhered to the culture bottle. Every 48 h, the culture medium was changed after washing the T-25 culture bottle with sterile PBS. When the cells reached 90% confluence, cells in the T-25 culture bottle were trypsinized. As the cells dislodged from the T-25 culture bottle, 4 ml of culture medium was added. This material was transferred to a 15-ml tube and centrifuged at 100×g for 5 min. The supernatant was discarded and 8 ml of culture medium was added to the pellet. This material was now transferred to a T-75 culture bottle and re-incubated in a CO₂ incubator at 37°C, with the culture medium being changed every 48 h. Further trypsinization was performed when the cells reached 90% confluence. Then, with each trypsinization (every 7 days), the material obtained was transferred to two bottles, the following week to four bottles, and the next week to eight bottles and so on, until we reached the number of cells needed for the experiment. On the day of the experiment, the T-75 bottles were washed with sterile PBS, trypsinized, centrifuged, and the pellet was resuspended in approximately 200 μL of Matrigel® (BD Biosciences, San Jose, CA, USA). At this time, SHED were in number of 2×10⁶ and in passage from 8 to 9.

Surgical procedure and study groups

Seventeen rats were distributed randomly into two groups. As the techniques differed as described below, the surgeon was not blinded to the study group. The surgery was carried out under 40× magnification by the aid of a surgical microscope (Carl Zeiss, Jena, Germany). Each animal was anesthetized and had the mandibular branch of the left facial nerve exposed and transected twice, generating one 5-mm nerve fragment that was employed as the autograft by suturing it with six isolated, epineural stitches using Nylon 10-0® monofilament and a BV-7 needle (Ethicon, Johnson & Johnson, New Brunswick, NJ, USA) and maintaining the previous orientation (Fig. 1).

The two study groups differed according to the surgical technique employed to conduct the facial nerve repair. Group-A animals comprised the control group (auto-graft). For animals in group B, the autologous graft was involved in a PGAt (GEM, Neurotube®, Synovis Micro, Birmingham, AL, USA) that measured 2.3 mm (inner diameter) by 6 mm (length). For this step, the neurotube was placed surrounding the proximal stump, followed by the suture of the graft. The tube was slid towards the graft and sutured to the perineural tissue with a single stitch with a nylon 10-0® monofilament and a BV-7 needle. The portion between the graft and the internal surface of the neurotube was subsequently filled with 200 μL of Matrigel® (BD Biosciences) disposed by the micropipette and the sterile tip, which contained 4×10⁵ of undifferentiated SHED. The ends of the PGAt were sealed with fibrin-derived biologic glue (Evicel®, Ethicon, Johnson & Johnson).

Functional analyses

Functional analyses were performed at three times points: before injuring the nerve and at 3 and 6 weeks after the surgical procedure. Animals were deeply anesthetized with
ketamine and submitted for neurophysiological evaluation by electromyography of the mandibular branch of the facial nerve aiming at obtaining the CMAPs. The CMAP amplitude values were the outcome variables.

To obtain CMAPs, we used a portable electromyography system (Neuro-MEP-Micro®, Neurosoft, Dhaka, Bangladesh) connected to a battery-operated Pavilion dv5c portable personal computer (Hewlett-Packard, Palo Alto, CA, USA). The Neuro-MEP.NET software (version 2.4.23.0, Neurosoft) was used to assess the CMPA data obtained under the following configuration of the electromyography system: 10-Hz high-pass filter, 10-kHz low-pass filter, notch filter off, 60 mV of leading edge signal, and 10-kHz of sampling rate. The electromyography protocol, modified from the protocol by Salomone et al., has been established specifically for the evaluation of the rat facial nerve.14

Histological analyses
Histomorphometric analyses were performed in a blinded fashion 6 weeks after the surgical procedure, and this method was well established by Costa et al.6,15,16. After the animals were sacrificed, the mandibular branch of the facial nerve was cut into two parts at a point 2 mm distal to the graft, and the proximal and distal fragments were analyzed at this point. The distal segment was fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.0031 M phosphate buffer, pH 7.3. After 60 min in this solution, the tissue was postfixed for 2 h in 2% osmium tetroxide in phosphate buffer, dehydrated in ethanol, infiltrated in propylene oxide and included in Epoxy® resin (Burlington, VT, USA) until polymerization. Transverse, 1-μm sections were made and stained with 1% toluidine blue. Histological observations were carried out using light microscopy (Nikon Eclipse E 600, Nikon, Tokyo, Japan). The slides were photographed with a digital camera (Nikon Coolpix E 955), and cell measurements were taken (Sigma Scan Pro 5.0 software, SPSS Science, Chicago, IL, USA). For quantitative analyses of the distal portion of the facial nerve, axons were counted in a partial area of 90,000 μm² in three random microscopic fields for every fiber displaying its center within it. The total axon density was obtained by the ratio between the total axonal number and the area. The shortest external diameter (including the myelin sheath) of all axons within a partial, randomly selected area (30,000 μm²) of the transversal section of the nerve was measured to evaluate the maturation of the myelinated fibers (Fig. 2).

The proximal part was fixed in 4% paraformaldehyde in phosphate-buffered saline. After fixation for 24 h, the tissue was embedded in paraffin and was prepared for microscopy. Sections (8 μm) were prepared using an 820-II microtome (Reichert-Jung, Vienna, Austria). Following xylol-based paraffin removal and tissue rehydration, three 10-min incubations in 3% hydrogen peroxide occurred. Immunofluorescence assays were carried out following standard protocols.17 Once transferred to the phosphate-buffered saline solution, the slides were incubated at room temperature for 16 h with anti-S100 (detection of Schwann cells) antibody (Abcam, Cambridge, MA, USA) raised in rabbit or anti-lamin A/C antibody (detection of transplanted human cells) raised in mouse (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), each in a 50-fold dilution. Importantly, the anti-lamin A/C is specific for the human antigen. After two 10-min washes, the slides were incubated with secondary antibodies directed to the Fc portion of rabbit and mouse antibodies, conjugated respectively to Alexa 488 and Alexa 596 (Jackson Immunoresearch, West Grove, PA, USA). After washing, the slides were assembled into

![Fig 2. Histological observations](image-url)

The mandibular branch of the facial nerve stained with 1% toluidine blue and observed using light microscopy (Nikon Eclipse E 600, Nikon, Japan). (A) Control group. (B) Group treated with SHED. Scale bar: 10 μm.
Table 1. Description of Axonal Density (µm²) and Diameter (µm) According to the Groups and Results of the Comparative Test (Kruskal-Wallis test).

| Groups          | Autograft (N = 7) | SHED (N = 10) | p     |
|-----------------|-------------------|---------------|-------|
| Axonal density  |                   |               | 0.004 |
| mean ± SD       | 0.021 ± 0.003     | 0.014 ± 0.004 |       |
| median           | 0.022 (0.015; 0.023) | 0.014 (0.006; 0.02) |       |
| Axonal diameter  |                   |               | <0.001|
| mean ± SD       | 2.13 ± 0.07       | 3.04 ± 0.49   |       |
| median           | 2.15 (2.04; 2.21)  | 3.01 (2.34; 3.92) |       |

According to Table 3, the group treated with SHED had a statistically significant reduction in the CMAP amplitude from the preoperative moment to 3 weeks (p < 0.001), followed by an increase, not statistically significant, in the amplitude between 3 and 6 weeks (p = 0.912). In the group treated with SHED, there was no statistically significant difference between means from the preoperative time and 6 weeks after the surgical protocol (p = 0.612), showing that at the end of the experiment the neural function was basically similar to the preoperative moment. In the control group, there was a reduction in the CMAP amplitude when the preoperative values were compared with values obtained either at 3 or 6 weeks after surgery (p < 0.05), with no statistically significant difference from 3 to 6 weeks (p>0.999).

In immunofluorescence assays, the human tongue was used as a positive control for lamin A/C staining as the antibody employed was specific for the human antigen. Human tongue tissue was also a negative control for the Schwann cell marker S100. The proximal and distal segments of the tubulized mandibular branch of the facial nerve were positive for S100. A few cells (Fig. 3, arrows) in the distal segment of the nerve were stained with the human-specific antibody against lamin A/C, showing a perinuclear distribution as expected.

Discussion

Stem cells from dental pulp have clinical advantages when compared with mesenchymal stem cells derived from bone marrow due to the simplicity and low morbidity of extraction and the higher proliferation rates in vitro. Dental pulp stem cells can be isolated from deciduous, permanent, or supranumerary teeth. These different stem cell populations have been isolated and studied since their initial discovery in the last decade. All of these populations have certain common characteristics: fibroblast-like morphology, high efficiency of forming adherent colonies, and high rates of proliferation in vitro.

SHED have self-renewal capacity, and most of them express markers of bone marrow stromal stem cells (CD90, CD73, and CD105), neural progenitors (Doublecortin, GFAP, and Nestin), oligodendrocytes, and immature neural cells (βIII-tubulin, A2B5, and CNPase), but not markers of mature oligodendrocytes (MPB and APC). Locally, SHED induce neoangiogenesis, differentiation into support cells such as Schwann cells, and facilitate axonal regeneration. The culture of SHED have been observed to produce neurotrophic factors such as neural growth factor, brain neurotrophic factor, and glial cell derived neurotrophic factor. This production of neurotrophic factors has shown therapeutic benefits in animal models of myocardial infarction, systemic lupus erythematosus, colitis, and cerebral ischemic injury. Of special interest for the present study, SHED have been demonstrated to differentiate in vitro in Schwann-like cells. Specific protocols have additionally...
been developed to obtain Schwann-like cells upon in vitro differentiation of mesenchymal stem cells from distinct origins, such as the bone marrow\textsuperscript{7,15}. The stem cell bank is feasible and a reality for SHED in countries such as Japan, Norway and India for the features mentioned above, including ease of production, ability to differentiate even after cryopreservation, and low immunogenicity potential. In addition, their immunomodulation potential of T cell immunosuppression is superior to that observed in bone marrow stem cells\textsuperscript{18,19}.

In 2011, Sasaki et al. evaluated facial nerve regeneration in rats treated with neuroconduit and SHED by the degree of neural attachment after configuring a 7-mm gap and tubulization with or without SHED\textsuperscript{20,21}. In the first study, silicone neuroconduit was used, and neural reconnection was evaluated 12 days after the procedure. In the second study, the polyglycolic acid neuroconduit was used, with analysis of neural reconnections performed on day 5 postoperatively. In both cases, the SHED group was observed to have a faster regeneration than in the control group. Comparing the two types of conduits, the silicone was not absorbable and thus generated a local chronic inflammatory process and pain if not removed. The polyglycolic acid neuroconduit is absorbable and thus did not cause the abovementioned complications, avoiding the need for reoperation\textsuperscript{20,21}.

In 2012, Dai et al. compared the use of Schwann cells (SC), adipose multipotent stem cells (ASC), dental pulp stem cells (DPSC), SC+ASC, and SC+DPSC in the repair of rat sciatic nerve lesions\textsuperscript{22}. The best functional result was observed in the SC+ASC combination, but all of the groups treated with cells showed superior functional results compared with the groups treated with neuroconduits alone. Thus, it was observed the presence of stem or Schwann cells to be beneficial in neural regeneration\textsuperscript{22}.

In our study, the functional evaluation demonstrated a better recovery of the CMAP amplitude in the SHED group compared with the control group. As expected, the CMAP amplitudes were reduced after 3 weeks in both groups due to the initial regenerative process at the time of the study. However, at 6 weeks, the group treated with SHED presented with a better functional response such that no statistically significant difference was observed between the end of the study and the preoperative phase. The CMAP amplitude at 6 weeks was essentially similar to the reference values.

Histological analyses showed that the SHED group had a larger mean diameter and a lower mean axonal density

| Table 2. Preoperative, 3- and 6-Week CMAP (mV) Amplitude Values. |
|---------------------------------------------------------------|
| Group | Autograft (N = 7) | SHED (N = 10) | \( p \) Group | \( p \) Moment | \( p \) Interaction |
|-------|------------------|---------------|--------------|---------------|------------------|
| Pre-operative | Mean ± SD | 5.86 ± 2.48 | 5.61 ± 2.28 | <0.001 | <0.001 | 0.034 |
| | Median | 6.25 (1.76; 9.35) | 5.05 (2.23; 9.72) | |
| 3 weeks | Mean ± SD | 0.39 ± 0.22 | 1.95 ± 1.9 | |
| | Median | 0.34 (0.14; 0.83) | 1.54 (0.59; 7.02) | |
| 6 weeks | Mean ± SD | 0.75 ± 0.46 | 3.79 ± 1.74 | |
| | Median | 0.7 (0.23; 1.53) | 2.9 (2.1; 7.36) | |

| Table 3. Bonferroni Multiple Comparisons of the CMAP Amplitude Between the Groups and Moments of Evaluation. |
|---------------------------------------------------------------|
| Group/ Moment | Comparison | Mean difference | \( p \) | CI (95%) |
|----------------|------------|-----------------|------|---------|
| Autograft Pre-op | 3 weeks | 5.47 | <0.001 | 2.46 | 8.47 |
| | 6 weeks | 5.11 | <0.001 | 2.28 | 7.93 |
| | 3 weeks | -0.36 | >0.999 | -3.37 | 2.64 |
| SHED Pre-op | 3 weeks | 3.66 | <0.001 | 1.15 | 6.18 |
| | 6 weeks | 1.82 | <0.001 | -0.54 | 4.19 |
| | 3 weeks | -1.84 | >0.999 | -4.35 | 0.68 |
| Pre-op Autograft SHED | | 0.25 | >0.999 | -2.37 | 2.87 |
| | 3 weeks | | | | |
| | 6 weeks | | | | |
| Pre-op Autograft SHED | | -1.56 | >0.999 | -4.18 | 1.06 |
| | 6 weeks | | | | |

Pre-op: Pre-operative; SHED: stem cells from human exfoliated deciduous teeth.
Fig 3. Immunofluorescence staining detects human lamin A/C-positive staining in the distal segment of the facial nerve. Human tongue was used as a positive control for lamin A/C and negative control for the Schwann cell marker S100. Proximal and distal segments of the tubulized mandibular branch of the facial nerve were positive for S100. A few cells (arrows) in the distal segment of the nerve were stained with the human-specific antibody against lamin A/C, which was in the perinuclear area. DNA is recognized by 4',6-diamidino-2-phenilindol-dihydrochloride (DAPI). Confocal images were acquired after z-sectioning in a Zeiss Laser Scanning Microscope LSM880 (Zeiss, Jena, Germany). Pseudocolors were obtained by the software ZEN (Zeiss Efficient Navigation, Jena, Germany). Scale bar: 20 μm.
compared with controls, thereby demonstrating the superiority of the group treated with SHED.

According to Schmalbruch, the axonal diameter is the best parameter for the analysis of neural regeneration because it corresponds directly to fibers with greater myelin sheath, which, consequently, are more effective and mature. In addition, Titmus and Faber demonstrated in 1990 that the axonal diameter is a reliable variable for the evaluation of neural regeneration and functional prognosis by the direct relation observed between the neural conduction velocity and the probability of target organ innervation.

The findings of higher axonal density in the autograft group may correspond to multiple and non-effective innervations, a phenomenon termed axonal sprouting. In 2012, Costa et al. described a model for quantitative histological analysis of the mandibular branch of Wistar rats in the facial nerve, where a mean partial density of 0.018 ± 0.002 axons/μm² in the distal axon samples in an area of 90,000 μm² was found. Thus, the values observed in the control group in this study, 0.021 ± 0.003 axons/μm², were superior to those described in normal nerves. The SHED group had a density of 0.014 ± 0.004 axons/μm², demonstrating lower values than normal and were compatible with the histology of an injured and regenerative nerve. Thus, higher values of axonal density do not show a correlation with effective neural regeneration, which is related to the axonal sprouting process, a fact that explains the higher density observed in the control group.

In 2013, Costa et al. also evaluated the functional and histological effects of BMSC combined with PGA in autografted rat facial nerves using the same methods of this study. After neuromatosis of the mandibular branch of the rat facial nerve, surgical repair consisted of nerve autograft (control group), nerve autograft with a conduit and did not observe viable cells at the end of the experiment. In the present study, we observed a greater versatility of SHED, since they integrated the neural tissue and modified its phenotype in the microenvironment possibly following the demand for neural regeneration.

The experimental model using the mandibular branch of the facial nerve in Wistar rats was chosen because these animals are easy to handle and have anatomical and histological parameters that have been well described in the literature. In addition, it is a widely accepted model for nerve repair and comparative neuroscience and genome expression studies.

In regard to the surgical technique, the autograft is currently the gold standard procedure for severe facial nerve lesions. Previous work by Costa et al. in 2013, as mentioned above, used the same methodology as in the present study and showed no functional or histological difference when comparing the autograft technique and the one that adds the use of polyglycolic acid neurotubes. Therefore, because the technique used as a gold standard in the daily clinical routine showed functional and histological equivalence and a lower cost than that involving the neurotube, we chose to use only the autograft.

Bone marrow mesenchymal stem cells have already been shown to potentiate neural regeneration. However, SHED are shown to have a higher potential for being simpler with higher rates of cell proliferation in vitro and with a better potential for neural regeneration possibly because they are directly derived from the neural crest in vivo. In this study, we showed a potential role of SHED for in vivo differentiation more appropriately attending the demands of the microenvironment.

**Conclusion**

In conclusion, the regeneration of the mandibular branch of the facial nerve in Wistar rats was superior in the group
treated with SHED associated with polyglycolic acid neurorotube compared with the autograft group. SHED integrated and remained in neural tissue for 6 weeks since transplantation, with a cell marker expression pattern similar to that seen in Schwann-like cells, suggesting in vivo differentiation.

Acknowledgments
RCMN, DBC, and LAH acknowledge funding from São Paulo Research Foundation, FAPESP (CEPID 2013/08028-1) through the facilities of the Human Genome and Stem Cell Research Center (Instituto de Biociências, University of São Paulo, Brazil). This research was funded by FAPESP grants (2014/18764-0).

Ethical Approval
Ethical approval for this original research was obtained by Administration Committee of Experimental Animals, University of São Paulo, Brazil (no.075/14).

Statement of Human and Animal Rights
All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of University of São Paulo, Brazil and approved by Administration Committee of Experimental Animals, University of São Paulo, Brazil (no.075/14).

All the experimental procedures involving humans were approved by the Ethics Committee, Biosciences Institute, University of São Paulo, Brazil (no.711.639/14).

Statement of Informed Consent
Written informed consent was obtained from legally representative(s) for anonymized patient information to be published in this article.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Coordination for the Improvement of Higher Education Personnel, CAPES; Brasilia, DF, Brazil) finance code 001 and by São Paulo Research Foundation, FAPESP (CEPID 2013/08028-1 and 2014/18764-0). The co-author Dayane B. Cruz also received CAPES funding.

ORCID iD
Larissa Vilela Pereira https://orcid.org/0000-0003-3284-5641
Ricardo Ferreira Bento https://orcid.org/0000-0003-3749-4684

References
1. Bento RF, Miniti A. Anastomosis of the intratemporal facial nerve using fibrina tissue adhesive. Ear Nose Throat J. 1993; 72:663–672.
2. Bento RF, Miniti A, Ruocco JR. Traumatic peripheral facial palsy. In: Portmann M, editor. Proceedings: 5th Facial Nerve Symposium, Bordeaux, 1984. Masson: Paris; 1985. p. 299–303.
3. Spector JG, Lee P, Derby A. Rabbit facial nerve regeneration in autologous nerve grafts after antecedent injury. Laryngoscope. 2000;110(4):660–667.
4. Darrouzet V, Duclos JY, Liguoro D, Trulhle Y, Bonfils C, Bebear JP. Management of facial paralysis resulting from temporal bone fractures: our experience in 115 cases. Otolaryngol Head Neck Surg. 2001;125(1):77–84.
5. Costa HJZR. Estudo Experimental Sobre A Regenerac¸a˜oP o´s-Traumática Do Nervo Facial Em Coelhos. São Paulo: Faculdade de Ciências Médicas da Santa Casa de São Paulo; 2003.
6. Costa HJZR, Salomone S, Silva CF, Costa MP, Ramos MBLR, Bento RF. Quantitative histological analysis of the mandibular branch of the facial nerve in rats. Acta Cirurgica Bras. 2012; 27(11):747–750.
7. Costa HJ, Bento RF, Salomone R, Azzi-Nogueira D, Zanatta DB, Paulino Costa M, da Silva CF, Strauss BE, Haddad LA. Mesenchymal bone marrow stem cells within polyglycolic acid tube observed in vivo after six weeks enhance facial nerve regeneration. Brain Res. 2013;1510:10–21.
8. Salomone R, Bento RF, Costa HJ, Azzi-Nogueira D, Ovando PC, Da-Silva CF, Zanatta DB, Strauss BE, Haddad LA. Bone marrow stem cells in facial nerve regeneration from isolated stumps. Muscle Nerve. 2013;48(3):423–429.
9. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9: 641–650.
10. Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H. Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur J Neurosci. 2001;14(11):1771–1776.
11. Satar B, Karahatay S, Kurt B, Ural AU, Safali M, Avcu F, Oztas E, Kucukat Z. Repair of transected facial nerve with mesenchymal stromal cells: histopathologic evidence of superior outcome. Laryngoscope. 2009;119(11):2221–2225.
12. Keilhoff G, Fansa H. Mesenchymal stem cells for peripheral nerve regeneration - A real hope or just an empty promise? Exp Neurol. 2011;232(2):110–113.
13. Nakamura S, Yamada Y, Katagiri W, Sugito T, Ito K, Ueda M. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J Endod. 2009; 35(11):1536–1542.
14. Jarmalavičius A, Tunaitis V, Strainienė E, Aldonytė R, Ramanavičius A, Venalis A, Magnuson KE, Pivoriūnas A. A New Experimental Model for Neuronal and Glial Differentiation Using Stem Cells Derived from Human Exfoliated Deciduous Teeth. J Mol Neurosci. 2013;51:307–317.
15. Dezawa M. Central and peripheral nerve regeneration by transplantation of Schwann cells and transdifferentiated bone marrow stromal cells. Anat Sci Int. 2002;77:12–25.
16. Salomone R, Costa HJZR, Rodrigues JRF, Reis e Silva SM, Ovando PC, Bento RF. Assessment of a neurophysiological model of the mandibular branch of the facial nerve in rats by electromyography. Ann Otol Rhinol Laryngol. 2012;121(3):179–184.
17. Costa HJZR, Silva CF, Costa MP, Lazarini PR. Evaluation of the systemic use of riluzole in post-traumatic facial nerve regeneration: experimental study on rabbits. Acta Otolaryngol. 2007;127(11):1222–1225.
18. Kerkis I, Caplan AI. Stem cells in dental pulp of deciduous teeth. Tissue Eng. 2012;18(2):129–138.
19. Didilescu AC, Rusu MC, Nini G. Dental pulp as a stem cell reservoir. Rom J Morphol Embryol. 2013;54(3):473–478.
20. Sasaki R, Aoki S, Yamato M, Uchiyama H, Wada K, Okano T, Ogiuchi H. Tubulation with dental pulp cells promotes facial nerve regeneration in rats. Tissue Eng. 2008;14(7):1141–1147.
21. Sasaki R, Aoki S, Yamato M, Uchiyama H, Wada K, Ogiuchi H, Okano T, Ando T. PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration. J Tissue Eng Regen Med. 2011;5(10):823–830.
22. Dai LG, Huang GS, Hsu SH. Sciatic nerve regeneration by cocultured Schwann cells and stem cells on microporous nerve conduits. Cell Transplant. 2013;22(11):2029–2039.
23. Costa HJZR, Silva CF, Korn GP, Lazarini PR. Posttraumatic facial nerve regeneration in rabbits. Brazilian J Otorhinolaryngol. 2006;72(6):786–793.
24. Oiticica J, Barboza-Junior LC, Batissoco AC, Lezirrovitz K, Mingroni-Netto RC, Haddad LA, Bento RF. Retention of progenitor cell phenotype in otospheres from guinea pig and mouse cochlea. J Tansl Med. 2010;8:119.
25. Titmus MJ, Faber DS. Axotomy-induced alterations in the electrophysiological characteristics of neurons. Prog Neurobiol. 1990;35(1):1–51.
26. Schmalbruch H. Fiber composition of the sciatic rat nerve. Anat Rec. 1986;215(1):71–81.