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

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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[1]. Severe facial nerve lesions (neurotmesis) do not result in spontaneous recovery, and functional outcomes are still poor even after the best surgical repair[2–4].

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[5–8]. Positive effects with bone marrow stem cells (BMSC) on facial nerve regeneration have been demonstrated, but there have been only a few studies that

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employed stem cells from dental pulp for facial nerve repair[7–12]. Dental pulp appears to be an alternative and less invasive source of stem cells when compared with BMSC[13]. 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[9–13]. 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[9–13].

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 cm^2 culture flasks (Corning). Cells were kept at 37°C in a 5% CO_2 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 (FACSAria II - BDBiosciences, San Jose, CA, USA). Cells were harvested with Tryple Express, and resuspended to 10^5 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 5×10^3 cells/cm^2 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

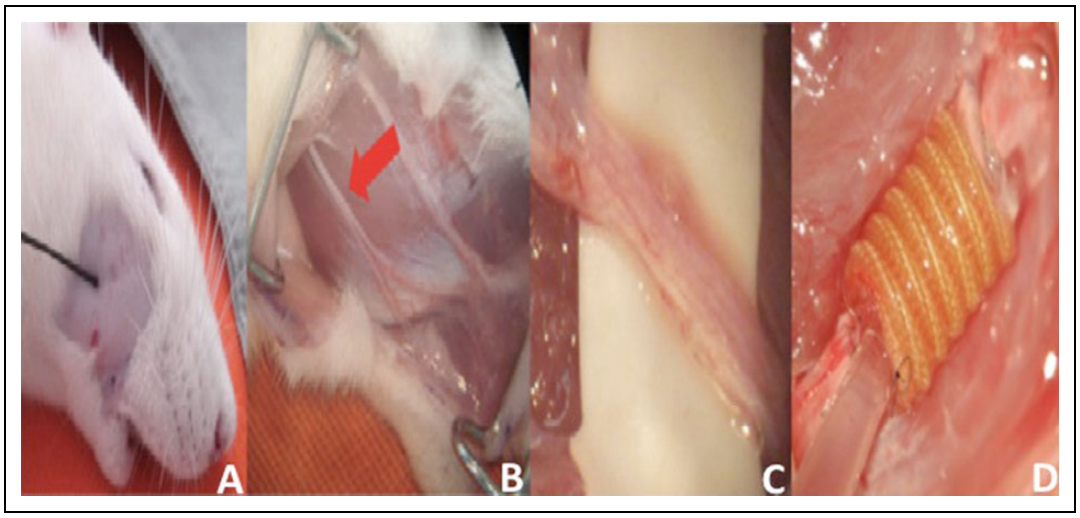

Fig 1. Surgical procedure. (A) Anesthetized animal had fur removed from the site of the intended incision before being submitted to the surgical procedure. (B) Dissection of the mandibular (red arrow) and buccal branches of the left facial nerve. (C) Isolation and transection of the buccal branch of the left facial nerve of the animal. (D) General aspect of the transected left facial nerve mandibular branch after grafting, neuroconduit, SHED transplantation and fibrin-derived biologic glue placement.

cryoprotective agent (DMSO). The Falcon tube was centrifuged for about 5 min at a rate of $100\times g$, 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 CO_2 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 $100\times g$ 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 CO_2 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 2×10^6 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 4×10^5 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

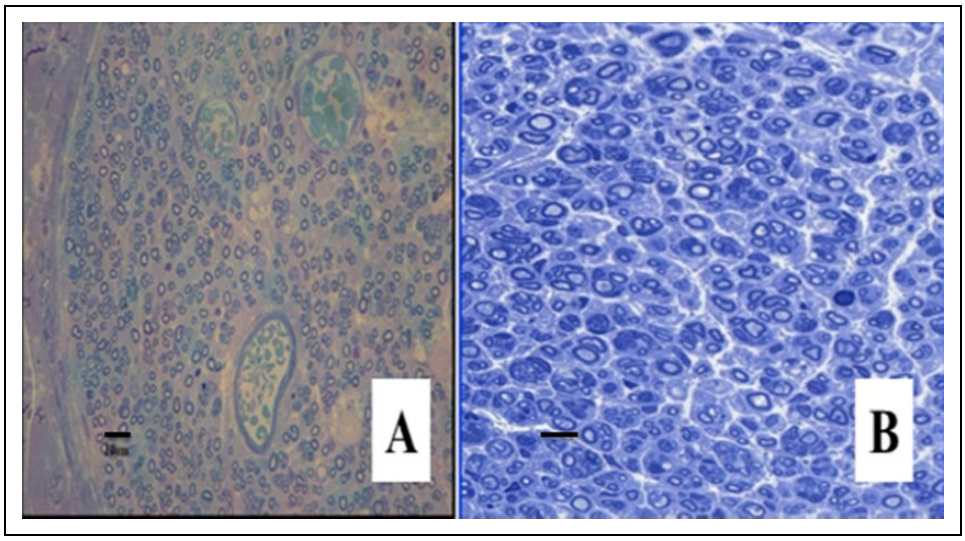

Fig 2. Histological observations The mandibular branch of the facial nerve stained with 1% toluidine blue and observed using light microscopy (Nikon Eclipse E 600, Nikon, Japan). (A) Control group. (B) Group treated with SHED. Scale bar: 10 μm.

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 μm^2 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 μm^2) 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

Table 1. Description of Axonal Density (μm^2) 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)	

4′,6-diamidino-2-phenyldindole-dihydrochloride (DAPI, Abcam) medium, which stained DNA. Confocal images were acquired after *z* sectioning in a Zeiss Laser Scanning Microscope LSM880 (Zeiss). Pseudocolors were obtained by the ZEN software (Zeiss Efficient Navigation, Jena, Germany).

Statistical analyses

The sample size was calculated based on similar previous studies, assuming a statistical power of 80% and α value of 5%.

The means obtained for the CMAP amplitude for each group were analyzed by generalized estimation equations with a matrix of 1-order autoregressive correlations between moments, with normal marginal distribution and identity bond function. Bonferroni multiple comparisons were used to determine the differences in amplitude among the groups and moments. The axonal density and diameter were compared by the Kruskal-Wallis tests followed by Dunn comparisons. All statistical analyses were performed using the SPSS® software, version 20.0®. The significance level was 5% ($p = 0.05$) unless when adjusted by the Bonferroni coefficient.

Results

As seen in Table 1, both the axonal density and diameter differed statistically between groups A and B, with higher values of axonal density in the control group ($p = 0.004$), and the largest axonal diameter in the group treated with SHED ($p < 0.001$).

CMAP amplitudes were significantly different between groups when the preoperative time values were compared with those from the 6-week postoperative time. In the autograft group, the mean CMAP amplitude at 6 weeks after surgery was equivalent to 13% of the preoperative CMAP amplitude. In the group treated with SHED, the mean CMAP at 6 weeks corresponded to 68% of the preoperative values (Table 2).

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*[18,19].

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)[18,19]. 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[18]. Of special interest for the present study, SHED have been demonstrated to differentiate *in vitro* in Schwann-like cells[14]. Specific protocols have additionally

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			<0.001	<0.001	0.034
Mean ± SD	5.86 ± 2.48	5.61 ± 2.28			
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.

					CI (95%)	
Group/ Moment	Comparison		Mean difference	p	Inferior	Superior
Autograft	Pre-op	3 weeks	5.47	<0.001	2.46	8.47
	Pre-op	6 weeks	5.11	<0.001	2.28	7.93
	3 weeks	6 weeks	-0.36	>0.999	-3.37	2.64
SHED	Pre-op	3 weeks	3.66	<0.001	1.15	6.18
	Pre-op	6 weeks	1.82	0.612	-0.54	4.19
	3 weeks	6 weeks	-1.84	0.912	-4.35	0.68
Pre-op	Autograft	SHED	0.25	>0.999	-2.37	2.87
3 weeks	Autograft	SHED	-1.56	>0.999	-4.18	1.06
6 weeks	Autograft	SHED	-3.04	0.006	-5.66	-0.42

Pre-op: Pre-operative; SHED: stem cells from human exfoliated deciduous teeth.

been developed to obtain Schwann-like cells upon *in vitro* differentiation of mesenchymal stem cells from distinct origins, such as the bone marrow[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[18,19].

In 2008 and 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[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[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[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[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

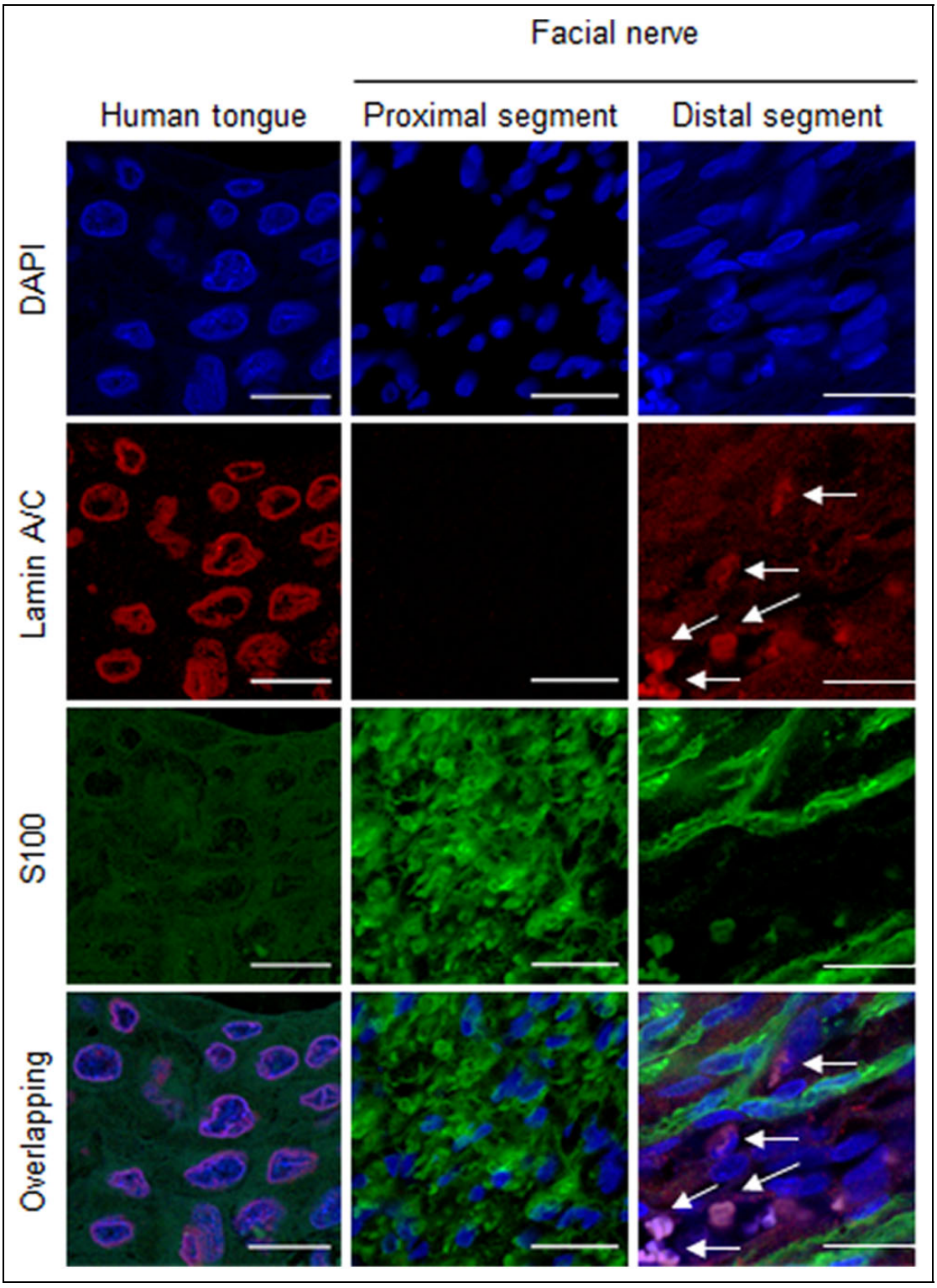

Fig 3. Immunofluorescence staining detects human lamin A/C-positive staining in the distal segment of the facial nerve. Human tongue was used as a positive control for lamin A/C and negative control for the Schwann cell marker S100. Proximal and distal segments of the tubulized mandibular branch of the 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[26]. 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[17,23].

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/μm^2 in the distal axon samples in an area of 90,000 μm^2 was found. Thus, the values observed in the control group in this study, 0.021 ± 0.003 axons/μm^2, were superior to those described in normal nerves. The SHED group had a density of 0.014 ± 0.004 axons/μm^2, 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[6].

In 2013, Costa et al. also evaluated the functional and histological effects of BMSC combined with PGAt in autografted rat facial nerves using the same methods of this study[7]. After neurotmesis of the mandibular branch of the rat facial nerve, surgical repair consisted of nerve autografting (groups A–E) contained in PGAt (groups B–E), filled with basement membrane matrix (groups C–E) with undifferentiated BMSC (group D, uBMSC) or Schwann-like cells that had differentiated from BMSC (group E, dBMSC). The CMAP amplitude analyses made in the 6-week postsurgical point revealed differences among the five groups (0.74, 0.76, 0.99, 1.96, and 2.73 mV, respectively) for groups A through E. A statistically significant difference was found between cell-containing groups (D and E) versus autograft (A) or autograft and neurotube (B) ($p = 0.004$). In quantitative histological analyses, groups A through E presented with increasing mean axonal diameters (2.17, 2.13, 2.73, 3.07, and 3.59 μm, respectively) and with an axonal density of 0.0214, 0.0171, 0.0208, 0.0151, and 0.0172 axons/μm^2, respectively. These data indicate that the CMAP amplitude was significantly higher for uBMSC and dBMSC 6 weeks after surgery, but the values were inferior when compared with the values obtained in the DPSC group in the present study (3.79 ± 1.74 mV). Histological analyses showed better results in the uBMSC and dBMSC groups with higher axonal diameters and inferior axonal densities. We also showed that the axonal diameter was 3.04 ± 0.49 μm and the mean axonal densities was 0.014 ± 0.004 in the SHED group. Therefore, our study disclosed better functional (CMAP amplitude) results in the SHED group as compared with the uBMSC or dBMSC groups from the report by Costa et al. with similar histological features[7].

Immunofluorescence showed the expression of human lamin A/C and S100 markers within the neuroconduit, indicating that viable SHED are present after 6 weeks of transplantation, with a marker expression phenotype seen in Schwann-like cells. We demonstrated the presence of viable and active SHED in neural regeneration even at the end of the protocol with differentiation into Schwann cells *in vivo*. The viability and differentiation of SHED was likely possible by the microenvironment and was more favorable for cellular survival that was provided by the absorbable polyglycolic acid neurotube.

In 2013, Costa et al. also revealed that BMSC integrated in the neural tissue but maintained the former cell phenotype for 6 weeks[7]. Salomone et al. (2013) used a silicone neuroconduit and did not observe viable cells at the end of the experiment[8]. 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[7,8,24,25].

In regard to the surgical technique, the autograft is currently the gold standard procedure for severe facial nerve lesions[1,3,4]. 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[7]. 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[7].

Bone marrow mesenchymal stem cells have already been shown to potentiate neural regeneration[7,8]. 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[18–21]. In this study, we showed a potential role of SHED for *in vivo* differentiation more appropriately attending the demands of the microenvironment[18,19].

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 neurotube 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.

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Ethical Approval

Ethical approval to 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.

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