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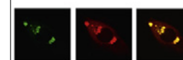

Research Report

Mesenchymal bone marrow stem cells within polyglycolic acid tube observed *in vivo* after six weeks enhance facial nerve regeneration

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ABSTRACT

Autografting is the gold-standard method for facial nerve repair with tissue loss. Its association with high-quality scaffolds and cell implants has disclosed distinct experimental outcomes. The aim of this study was to evaluate the functional and histological effects of bone marrow stem cells (BMSC) combined with polyglycolic acid tube (PGAt) in autografted rat facial nerves. 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) or Schwann-like cells that had differentiated from BMSC (group E). Axon morphometrics and an objective compound muscle action potentials (CMAP) analysis were conducted. Immunofluorescence assays were carried out with Schwann cell marker S100 and anti-β-galactosidase to label exogenous cells. Six weeks after surgery, animals from either cell-containing group had mean CMAP amplitudes significantly higher than control groups. Differently from other groups, facial nerves with Schwann-like cell implants had mean axonal densities within reference values. This same group had the highest mean axonal diameter in distal segments. We observed expression of the reporter gene *lacZ* in nerve cells in the graft and distally from it in groups D and E. Group-E cells had *lacZ* coexpressed with S100. In conclusion, regeneration of the facial nerve was improved by BMSC within PGAt in rats, yet Schwann-like cells were associated with superior effects. Accordingly, groups D and E had BMSC integrated in neural tissue with maintenance of former cell phenotype for six weeks.

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1. Introduction

Post-traumatic peripheral facial palsy is a debilitating condition with an increasing prevalence due to the high frequency of accidents and violence in modern life leading to facial asymmetry, impacting eye and oral motor functions, self-esteem and mood (Bento et al., 1985). Restoration of function after transection and repair of the facial nerve is still poor, leading to residual paralysis, sinkinesis and hypotonia (Bento and Miniti, 1993; Ferreira et al., 1994). Trauma and tumor surgical resection may result in extensive nerve damage, requiring repair by autologous nerve interposition grafting or tubulization (Da-Silva et al., 1987; Bento and Miniti, 1989), but full functional recovery is seldom achieved.

Nerve repair requires a complex interaction among a scaffold for axonal growth guidance, supportive cells such as Schwann cells, growth factors, and extracellular matrix (Da-Silva et al., 1985; Costa et al., 2006; Costa et al., 2009a). The combination of axonal scaffolds and transplanted cells provides adequate support for neural regeneration, and has been investigated as a strategy to overreach the limitations of surgical repair (Evans et al., 2002; Cheng and Chen, 2002; Udina et al., 2004; Rodrigues et al., 2012). In particular, the polyglycolic acid tube (PGAt), composed of absorbable material, has been established as an appropriate conduit for nerve grafting, and has been approved by the Food and Drug Administration (FDA, USA) for use in the clinical setting (Mackinnon and Dellon, 1986; Da-Silva et al., 1987; Mackinnon and Dellon, 1990; Weber et al., 2000; Costa et al., 2006; Costa et al., 2009b; Schlosshauer et al., 2006; Nectow et al., 2011).

Isolated and cultured Schwann or stem cells have been employed in the surgical repair of the peripheral nerve (Dezawa et al., 2001; Cuevas et al., 2002; Evans et al., 2002; Fansa and Keilhoff, 2004; Udina et al., 2004; McKenzie et al., 2006; Chen et al., 2007; Lavdas et al., 2008; Ishikawa et al., 2009; Wang et al., 2009; Wakao et al., 2010; Wei et al., 2010; Ladak et al., 2011; Wang et al., 2011; Rodrigues et al., 2012; Salomone et al., 2013). Schwann-like cells have been reported to differentiate *in vitro* from bone marrow stroma mesenchymal stem cells (BMSC) primarily cultured from rat femurs (Dezawa et al., 2001; Chen et al., 2007). Schwann-like cells experimentally employed in peripheral nerve repair have improved myelination (Dezawa et al., 2001; Cuevas et al., 2002; Chen et al., 2007; Ishikawa et al., 2009; Wang et al., 2011). Although there are limited data on the association of PGAt and genetically modified BMSC-derived Schwann-like cells in the repair of the facial nerve (Shi et al., 2009), a thorough, objective analysis on the functional nerve recovery and of *in vivo* cell survival is lacking.

Our approach in the current study has been to employ the gold-standard nerve repair procedure, nerve autografting, combined to bone marrow mesenchymal stem cells seeded in purified basement membrane as a secondary scaffold, used to fill the lumen of PGAt. Our aims were to compare the facial nerve functional and morphological outcomes, and to evaluate the presence and phenotype of the exogenous cells in the autografted nerve, six weeks after implantation.

The use of five different animal groups allowed for progressive addition of each component to be tested. An objective comparison was performed to assess compound muscle action potential (CMAP) and axonal morphometry variables. We disclose the highest CMAP amplitudes and axonal diameters in the Schwann-like cell autografted group. Our study also reveals unprecedented results on the *in vivo* maintenance of the stem cells for six weeks in the nerve tissue, which may be related to the superior characteristics of the conduit and extracellular membrane components employed.

2. Results

Prior to surgery, lentivirus-transduced BMSC (BMSC*lacZ*+) obtained *in vitro* reacted positively in the colorimetric assay for *lacZ* activity, whereas untransduced BMSC did not (Fig. 1, A and B). BMSC*lacZ*+ differentiated *in vitro* in cells that were immunostained for beta-galactosidase (Fig. 1, D, G and J), presented thin and long cell processes (Fig. 1, H and K, arrows), and expressed the cell markers S100, $p75^{NTR}$ and Oct6 in the nucleus and cytoplasm (Fig. 1, C, F and I) that were undetectable in undifferentiated cells.

At surgery, three animals from group E died most likely due to hypersensitivity to anesthesia maintenance. On the second day of the postsurgical period, one animal from group D died due to unexplained cause. Data that had been previously obtained for these animals were not considered in this study.

2.1. *Significant improvement of CMAP amplitude associated with cell implants*

Data analyses using the Kruskal–Wallis test disclosed no difference among groups regarding CMAP amplitude or latency prior to neurotmesis and three weeks after surgery (Fig. 2A). On the other hand, CMAP amplitude analyses made in the six-week postsurgical point revealed differences among the five groups (0.74 mA, 0.76 mA, 0.99 mA, 1.96 mA, 2.73 mA, respectively for groups A, B, C, D and E; $p<0.001$, Fig. 2A). Assessment by the Mann–Whitney test adjusted by the Bonferroni coefficient (alpha=0.005116) disclosed a difference between any control group without Matrigel® (A or B) and any group of cell-containing Matrigel® (D or E): $p=0.004$ for each comparison, A *vs.* D; A *vs.* E; B *vs.* D; and B *vs.* E (Fig. 2A). Other possible paired comparisons were not significant. These data indicate that CMAP amplitude is significantly higher for groups D and E six weeks after surgery. At the sixth week, groups D and E presented respectively 44.52% and 72.03% of their pre-injury CMAP amplitude values, whereas groups A, B and C had the ratios of 12.8%, 15.94% and 16.98% in the same period (Fig. 2A). Therefore, some functional recovery has been observed for each study group.

2.2. *Exogenous cells are maintained in vivo six weeks after surgery*

Qualitative histological analyses at the optical microscope of segments proximal and distal to the graft revealed that, in study groups A through D, the facial nerve has been reorganized in one to three fascicles in the distal segment, whereas group-E animals had the injured facial nerve reorganized in

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