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Original Research Article

# Micropuncture and pressure assisted Schwann cell seeding of nerve allograft

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## HIGHLIGHTS

- Schwann cell activity and migration deteriorates with increasing allograft length.
- Injection method appears to deliver a larger number of viable Schwann cells at 21 days compared to other bathing methods.
- Neither microneedle puncture nor pressurization seemed to offer a substantial benefit in Schwann cell seeding of nerve allograft over simply bathing.

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## ABSTRACT

*Background:* Tissue processing to create immunotolerant nerve allograft removes neurosupportive cells. Few strategies have been described for implanting new cells into the graft to support axonal regeneration.
*New method:* Micropuncture of the nerve allograft surface combined with immersion into a pressurized cell-rich solution to potentiate the introduction of viable Schwann cells (SC) into processed nerve allograft. Allografts were used to repair rodent sciatic nerve defects. At 3, 7, and 21 days, grafts were harvested, stained for SCs, and analyzed using total cross sectional area (CSA) occupied by SCs to quantify SC presence.
*Results:* At days 3 and 7, SC CSA was significantly greater for the injection group compared to all other groups. At day 21, SC CSA for the injection group ($0.2252\% \pm 0.2730$) was significantly greater compared to following groups: pressurized-punctured ($0.0653\% \pm 0.0934$), nonpressurized-nonpunctured ($0.0607\% \pm 0.0709$), punctured-control ($0.0246\% \pm 0.0398$), and nonpunctured-control ($0.0126\% \pm 0.0151$). A significant decrease in percent CSA occupied by SCs from day 3 to day 21 was noted in nonpressurized-punctured group ($p = 0.0106$), pressurized-nonpunctured group ($p = 0.0477$), and injection group ($p = 0.0010$).
*Comparison with existing method(s):* Most studies have used small caliber hypodermic needles to inject the cells into grafts.
*Conclusions:* Despite a presumed decrease in cell viability over the three weeks of the study, the large initial inoculum achieved by injection technique results in higher levels of final SC seeding in acellular nerve allograft compared with bathing techniques with or without micropuncture or pressurization.


## 1. Introduction

Commercially available acellular human nerve allograft is emerging as a useful tool in overcoming small and medium nerve gaps based on convenience, ease of use, and reasonable effectiveness. For critical large mixed motor nerve repairs and longer gaps, acellular allograft, however, has not gained as much popularity, in part due to a lack of the necessary support cells native to nerve

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autograft. Efforts to maximize the neurosupportive microenvironment within allograft by implanting exogenous neurosupportive cells offers a potential solution. While much research has focused on identifying which candidate cells to implant, less studies have been directed at the actual seeding or implantation process. The optimal seeding process should deliver high quantities of viable cells in an even distribution throughout the nerve allograft.

Schwann cells have several essential roles within nerve tissue ranging from maintaining aneurosupportive environment to phagocytizing cellular debris following axonotmetic injury (Feinberg et al., 1997; Sunderland, 1990; David and Lacroix, 2003). Most relevant to acellular nerve allograft, SCs provide essential neurotrophic support and guidance necessary for axonal elongation (Gordon, 2009). SCs proliferate and arrange in rows of cells known as Bands of Büngner before up-regulating growth factors and secreting a network of collagen and laminin to support regenerating axons (Evans, 2001; Gaudet et al., 2011).

Currently, the only clinically relevant nerve allograft (in the United States) is rendered immunotolerant and acellular using a patented detergent process. Though cellular debris is removed, native SCs must still migrate into and populate the allograft tissue from both in situ nerve stumps before axon regeneration can occur (Hudson et al., 2004). However recently, it has been demonstrated that SC activity and migration deteriorates with increasing allograft length and may offer a significant obstacle to acellular allograft treatment of longer nerve gaps (Saheb-Al-Zamani et al., 2013).

A theoretical solution to this dilemma would be to add Schwann (or "Schwann-like") cells to the allograft so that the allograft effectiveness was not as dependent on "native" SC migration. Cultured SCs and stem cells derived from bone marrow, adipose tissue, and skin have all been shown to improve axonal regeneration(Jesuraj et al., 2011; Zhang and Lv, 2013; Wang et al., 2012; Hu et al., 2007; Wang et al., 2010; Di Summa et al., 2011; Walsh et al., 2009).

Regardless of which cell type is being considered for allograft augmentation, the best method of populating the allograft with these cells has not been fully elucidated. Most studies have used small caliber hypodermic needles to inject the cells into the allograft. Potential disadvantages of this technique include damage to the internal architecture of the graft from either the needle or generated hydrostatic pressure. High shear stresses generated within the hollow needles also can damage the injected cells. The surviving cells must then compete for local diffused nutrients (the allograft is initially avascular) which may be inadequate especially within the center core of the graft or when cells clump together so that high initial cell counts may rapidly diminish.

Less traumatic and more uniform seeding techniques would seem desirable to achieve sustained levels of supportive cells. Bathing nerve allograft in a SC concentration and using multiple microneedle punctures through the outer epineurium combined with positive atmospheric pressure to "push cells" into the nerve tissue may offer alternative and advantageous strategies.

## 2. Materials and methods

### 2.1. Experimental design

Eighty-four rodent acellular nerve allografts measuring approximately 10 mm by 1.5 mm (prepared analogously to human acellular nerve allograft, AxoGen, Inc. Alachua, FL) were divided into 7 groups and prepared for implantation as outlined in Table 1. The first two groups tested the ability of positive pressure to improve access of the SCs into microneedle punctured allografts. Similarly, groups 3 and 4 tested the effects of pressure with allografts that had not been punctured. Groups 5 and 6 served as controls consisting of unseeded allografts with and without microneedle puncturing. In the final group, SCs were introduced into allografts via conventional microinjection.

### 2.2. Surgical procedure

Eighty-four three-month-old female Sprague-Dawley rats (300 g) were used in this study. All animal experiments were approved by the VCU Institutional Animal Care and Use Committee (IACUC) in accordance with the national guidelines and housed in a temperature and humidity controlled room with 12:12 h day: night cycle and with access to food ad libitum. Anesthesia was induced with 5% isoflurane administered via nose cone inhalation and maintained with 2–3% isoflurane. Core body temperature was maintained with a heating pad. Buprenorphine SR Lab (1–1.2 mg/kg) was administered subcutaneously for analgesia. Using aseptic technique, a standard bicep femoris semitendinosus muscle splitting approach was used to expose the left sciatic nerve and remove a 10 mm section centered midway between the sciatic notch and the bifurcation. A seeded or nonseeded (negative control) 10 mm rodent nerve allograft was sutured into the gap under microscope visualization with two to three 10–0 nylon epineurial stitches at each coaptation. The wound was then closed with 4–0 nylon sutures. The animals were maintained post-surgery for up to 21 days.

### 2.3. Cell culture

Commercially available rat SCs derived from postnatal day 8 rat sciatic nerve (Cat# R1700; ScienCell Research Laboratories, Carlsbad, CA) were propagated on $100 \times 20$ mm cell culture dishes coated with Poly-L-Lysine. Dulbecco's Modified Eagle Media (DMEM) supplemented with D-valine DMEM–d-valine; 2 mM glutamine; 10% (vol/vol) FCS; 1% (vol/vol) N2 Supplement; 20 μg/ml (wt/vol) bovine pituitary extract; 5 μM forskolin; 100 U/ml penicillin/100 μg/ml streptomycin; and 0.25/μg ml amphotericin B was used for propagation. Cells were grown to 75–90% confluence and then passaged up to a maximum of 5 passages. To prepare for administration to grafts, cells were trypsinized, counted and their concentration adjusted to $2 \times 10^8$ cells/mL in a total of 2.5 mL of medium.

### 2.4. Allograft preparation and cell seeding

Prior to inoculation with SCs, some allografts were micropunctured utilizing a commercially available microneedle roller (Risen Beauty Technology Co., Ltd., Beijing China, Model No DER100) comprised of five hundred and forty, 1.5 mm long by 0.1 mm diameter, titanium microneedles arranged in sixty rows of nine. Three passes were made with the microneedle roller before rotating the allograft a quarter turn and repeating until all four sides had been punctured. Some allografts did not undergo micropuncture. For seeding, allografts were incubated with the cell suspension for three minutes 1) under normal atmospheric pressure or 2) under 3 times atmospheric pressure (3 at.) using an air pump applied via a commercially available, round-bottom pressure flask with a thermowell port (Ace Glass, Vineland, NJ). As a positive control, one group of allografts underwent conventional "injection" seeding. The end of the allograft was temporarily secured and a 27-gauge Hamilton needle (Hamilton Company, Reno NV) was inserted down the center of the graft from the unsecured end. As it was withdrawn, 0.1 cc of labeled SC suspension was slowly injected into the allograft (Sun et al., 2009).