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Research article

# Tissue-engineered nerve graft with tetramethylpyrazine for repair of sciatic nerve defects in rats

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## HIGHLIGHTS

- Tissue-engineered nerve was constructed by acellular nerve allograft seeded with neural stem cells.
- Tetramethylpyrazine can effectively protect cells in the process of transplantation.
- Acellular nerve allograft has the similar three-dimensional structure compared to a normal autogenous nerve.

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## ABSTRACT

A tissue-engineered nerve with tetramethylpyrazine (TMP) was repaired for sciatic nerve defects in rats. A total of 55 adult Sprague Dawley (SD) rats were classified into 4 groups, with 15 rats in each of groups A, B, and C as well as 10 rats in group D. About 1.5 cm of a sciatic nerve of the right hind limb located 0.5 cm below the inferior margin of the piriformis was resected to form the defects. Four types of nerve grafts used for bridging nerve defects in the SD rats corresponded to the 4 groups: tissue-engineered nerves with TMP in group A, tissue-engineered nerves without TMP in group B, acellular nerve grafts (ANGs) in group C, and autologous nerves in group D. Twelve weeks post-surgery, the sciatic functional index, nerve conduction velocity, and gastrocnemius wet weight of groups A and D were higher than those of groups B and C ($P < 0.05$). Results of fluorescence microscopy and histological staining indicated that group A performed better than groups B and C ($P < 0.05$). Similarly, the number of horseradish peroxidase-labeled positive cells was significantly larger in group A than in groups B and C. Regenerative nerve fibers were abundant in group A and consisted mainly of myelinated nerve fibers, which were better than those in groups B and C ($P < 0.05$). The study demonstrated that tissue-engineered nerves constructed by ANGs seeded with neural stem cells and combined with TMP can effectively repair sciatic nerve defects in rats.



## 1. Introduction

Peripheral nerve injury occurs frequently in a clinical environment [1]. Autogenous nerve graft has been proven to be a distinct and reliable clinical method for treating nerve defects larger than 3 cm [2]. However, the clinical application of such a technique is restricted by the shortage of an autologous nerve, limitation of the nerve length and diameter for direct suturing, unavoidable secondary injury, and dysfunction of a donor nerve [3].

Studies on neuronal replacement have increasingly grown in recent years [4,5]. These studies indicate that acellular nerve grafts (ANGs) achieved an ideal result for the repair of a nerve defect smaller than 3 cm [6,7]. As a natural material, ANGs possess a similar, three-dimensional structure, and compared with a normal autogenous nerve; they are relatively easy to preserve and have extensive sources [8,9]. Studies [10–12] suggest that the ANGs reduce the immunogenicity and simultaneously retain the three-dimensional structure similar to a normal nerve.

Decline in the ability to promote axon regeneration is attributed to the reduced cell viability of the bridging graft [7]. Thus, the ANGs have to be combined with seed cells to exhibit low immunogenicity and then differentiate into functional nerve cells. Common seed cells contain Schwann cells (SCs) [13], bone marrow-derived mesenchymal stem cells (BMSCs) [14], and neural stem cells (NSCs)

*Abbreviations:* ANGs, acellular nerve grafts; NSCs, neural stem cells; TMP, tetramethylpyrazine.

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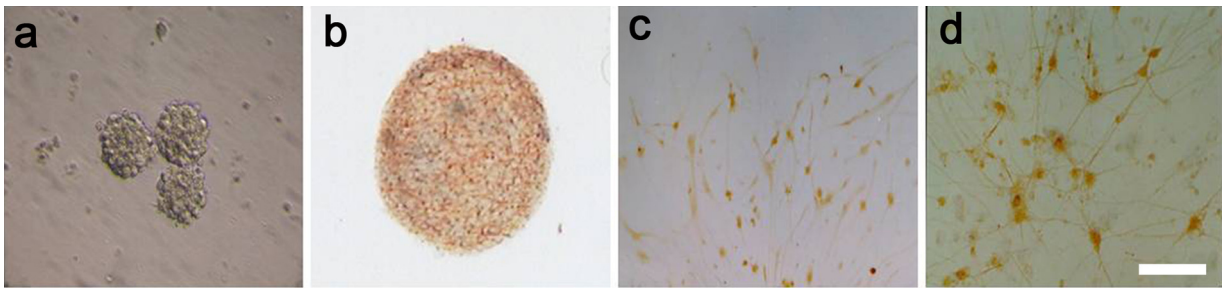


**Fig. 1.** a: Neural spheres after 5 days of culture, bar = 200 μm; b: Nestin, bar = 50 μm; c: NF 200, bar = 200 μm; d: GFAP, bar = 100 μm.

[15], among others. Studies have been successfully conducted on tissue-engineered nerve graft with SCs or BMSCs for the repair of peripheral nerve defects [16,17]. Nevertheless, a number of clinical problems have yet to be addressed. NSCs have been shown to exhibit low immunogenicity, good chemotaxis, and self-renewal [18]. NSCs can differentiate into neurons, which can influence the transmission of information, and oligodendrocytes, which participate in myelination [19].

Studies [20,21] indicate that tetramethylpyrazine (TMP) exhibits numerous pharmacologic actions, such as anti-inflammatory and anti-oxidant properties, apoptotic inhibition, reduction of oxygen glucose deprivation, and prevention of reperfusion injury. Our previous study shows that TMP plays an important role in cell proliferation, apoptotic inhibition, and protection of NSCs [22].

We hypothesized that ANGs seeded with cells can facilitate myelin regeneration, accelerate nerve conduction velocity, and promote the repair of sciatic nerve defects in rodents. Subsequently, we employed tissue-engineered nerve to repair the sciatic nerve defect in SD rats; ANGs with NSCs cultured in a medium containing TMP were combined. The purpose was to evaluate the effect of tissue-engineered nerve graft. We also aimed to investigate the repair effect of different grafts to identify better alternative materials for nerve graft.

## 2. Materials and methods

### 2.1. Animals

A total of 55 healthy Sprague Dawley (SD) rats (aged 2 months, weighing 200–250 g), 1 healthy female SD rat (15 days pregnant, weighing 300 g), and 30 healthy Wistar rats (aged 2 months, weighing 200–250 g) were examined. These experimental animals were provided by the Experimental Animal Center of Chongqing Medical University (Permit No. SCXK (Yu) 2007-0001).

### 2.2. Materials

Nestin antibody was purchased from Chemicon (United States). Neurofilament 200 (NF 200) antibodies were supplied by Dako Corp. (United States). Glial fibrillary acidic protein (GFAP) antibody was purchased from Shanghai Maixin. TMP powder (purity > 98%) was obtained from Sigma–Aldrich (United States).

## 3. Methods

### 3.1. ANGs co-cultured with NSCs to construct tissue-engineered nerve

An embryonic ependyma was obtained via dorsal sagittal incision from the head of fetal rats that were taken out from 15-day pregnant SD rats by Caesarean section after anesthetization and sterilization. It was then digested with trypsin to obtain a single-cell suspension. The cells were inoculated into 25 mL culture flasks in a serum-free DMEM/F12 (1:1) with a concentration of $5 \times 10^5$/mL as the basic medium supplemented with epidermal growth factor, basic fibroblast growth factor, and B27. The flasks were then placed in a constant-temperature oven (5% $CO_2$, 37 °C). The medium was centrifuged and half-exchanged every 3–4 days, and the cells were passaged every 7–10 days. The NSCs were identified by immunohistochemistry (Fig. 1).

The pLEGFP plasmid vector containing the enhanced green fluorescent protein gene was amplified, extracted, and transfected into the virus packaging cell PT67. The supernatant was collected, and the viral titer was determined. The supernatant was then used to transfect the NSCs and observe them under a fluorescent microscope. The transfected NSCs were cultured using the conventional method for 7 days to form neural spheres (Fig. 2).

Decellularization based on Hudson [23] and Sondell [24] was used to obtain acellular nerves from Wistar rats. During surgery, the rats remained anesthetized with 1% pentobarbital sodium (40 mg/kg); bilateral sciatic nerves were exposed in each rat. Fatty and connective tissues were removed from epineuria, sliced into 25 mm segments, and flushed with physiological saline. A total of 46 nerve segments were successively flushed with a 0.05 mmol/L Tris Hcl (pH 7.4) and a TritonX-100 (pH 7.4) phosphate buffer solution. Both solutions were added with protease inhibitors and then oscillated for 24 h at 4 °C. Each segment was then flushed with sterile distilled water and stored in PBS (pH 7.4) at 4 °C until use.

A total of 40 hydrated acellular nerve segments were injected with 15 μL ($1 \times 10^6$/mL) of genetically modified single-cell NSC suspension at both ends by using a micropipette. The acellular nerve segments were divided into groups I and II, with each group consisting of 20 samples. Group I was cultured in a conventional DMEM/F12 medium; group II was cultured in the same medium but with 200 mg/mL TMP added into it [22]. The segments were then placed in a constant-temperature oven (5% $CO_2$, 37 °C) for 1 week. The other acellular nerve segments were stored in PBS (pH 7.4) at 4 °C until use.

### 3.2. Experimental group

A total of 55 adult SD rats were classified into 4 groups; groups A, B, and C consisting of 15 rats each and group D with the remaining 10 rats. After the SD rats were anesthetized with 1% pentobarbital sodium (40 mg/kg) via abdominal injection, the sciatic nerves of their right hind limbs were exposed. About 1.5 cm of the nerve located 0.5 cm below the inferior margin of the piriformis was resected to form the defects. The defects were bridged with four kinds of materials, viewed under a surgical microscope, using 10-0 non-damage suture materials. A tissue-engineered nerve cultured in TMP (group II) was grafted in group A, a tissue-engineered nerve without TMP (group I) was grafted in group B, an acellular ner-